University of Alberta

Hepatic apolipoprotein B assembly and secretion

by

©

Sherri Lynn Selby

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ABSTRACT

Atherogenic lipoproteins secreted by the liver consist of apolipoprotein (apo) B, a neutral lipid core and an amphipathic monolayer primarily made up of phosphatidylcholine (PC). Apo B may be inefficiently translocated into the endoplasmic reticulum (ER) due to pause transfer sequences (PTS) distributed throughout the apo B protein. A region of apo B known to contain PTS was appended to a typical secretory protein and was tested to see if it might confer inefficient translocation, and subsequently be poorly secreted relative to the unadulterated protein, when expressed in hepatoma cells. Results indicated that the PTS containing protein was not secreted and instead formed aggregates. It was concluded that this model was not a suitable approach to address the role of PTS sequences in apo B containing lipoprotein secretion.

In the liver, PC can be synthesized from choline, from methylation of phosphatidylethanolamine (PE) synthesized from ethanolamine, or via methylation of PE derived from decarboxylation of phosphatidylserine (PS). Previous studies suggested that specific pools of phospholipids are used for lipoprotein secretion. However, the intracellular site of addition of PC derived from these various pathways to apo B containing lipoproteins had not been determined. Rats were injected with [^{3}H]choline via portal vein, the livers harvested, and ER and Golgi recovered after 30 min. Lumenal apo B associated PC was isolated to measure the radioactivity and mass. Results suggested that PC derived from choline might be added to apo B lipoproteins in the Golgi although the recovery of apo B from the ER lumen was incomplete. Further studies are required to establish the contribution and site of

ethanolamine and serine derived PC addition to apo B containing lipoproteins in the liver.

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LIST OF ABBREVIATIONS

3D, three dimensional

Å, angstroms

ABBP-1, apobec-1 binding protein-1

ABBP-2, apobec-1 binding protein-2

ACAT-1, coenzyme A:cholesterol acyltransferase-1

ACAT-2, coenzyme A:cholesterol acyltransferase-2

ACF, apobec-1 complementation factor

Apo B, apolipoprotein B

Apobec-1, apo B mRNA editing enzyme catalytic complex 1

ARP1, apolipoprotein AI regulatory factor 1

Asn, asparagine

AUX240, auxiliary protein 240

BAG4, Bcl-2-associated athanogene 4

BiP, immunoglobulin heavy chain binding protein

bp, base pair

BSA, bovine serum albumin

C terminus, carboxyl terminus

°C, degree Centigrade

C/EBP, CCAT/enhancer binding protein

CCT, CDP:phosphocholine cytidylyltransfrerase

CE, cholesteryl ester

CFS, cell free system

Cho, choline

CK, choline kinase

CM, chylomicron

COPII, coatamer protein complex II

CPT, choline phosphotransferase

CUGBP2, CUG binding protein

CXN, calnexin

Da, Daltons

DGAT-1, acyl coenzyme A:diacylglycerol acyltransferase-1

DGAT-2, acyl coenzyme A:diacylglycerol acyltransferase-2

DMEM, Dulbelco's modified Eagle's medium

Dpm, disintegrations per minute

ECT, ethanolamine cytidylyl transferase

EK, ethanolamine kinase

EPT, ethanolamine phosphotransferase

ER, endoplasmic reticulum

Etn, ethanolamine

G418, gentimycin

GATA-4, transcription factor that recognizes GATA sequences in DNA

GRY-RBP, 2glycine-arginine-tyrosine-rich RNA binding protein

H_2O, water

HDL, high density lipoprotein

HNF3, hepatic nuclear factor 3

HNF4, hepatic nuclear factor 4

hnRNP-C1, heterogeneous nuclear ribonucleoprotein-C1

Hsp, heat shock protein

ICR, intestinal control region

IDL, intermediate density lipoprotein

KSRP, KH-type splicing regulatory protein

LCAT, lecithin cholesterol acyltransferase

LCR, liver control region

LDL, low density lipoprotein

LPL, lipoprotein lipase

LRP, LDL-receptor related protein

MAM, mitochondrial associated membrane

MARs, matrix associated regions

MM, molecular mass

MME, monomethylethanolamine

uCi, microCurie

MTP, microsomal triacylglycerol transfer protein

N terminus, amino terminus

Na_2CO_3, sodium carbonate

nt, nucleotide

PBS, phosphate buffered saline

PC, phosphatidylcholine

PDI, protein disulfide isomerase

PE, phosphatidylethanolamine

PEMT, phosphatidylethanolamine methyltransferase

PEMT, phosphatidylethanoline methyl transferase

PMME, phosphatidylmonomethylethanolamine

PIP or post-IP, post-immunoprecipitation

Prl, prolactin

PS, phosphatidylserine

PSD, phosphatidylserine decarboxylase

PSS1, phosphatidylserine synthase 1

PSS2, phosphatidylserine synthase 2

PTS, pause transfer sequence

PVDF, polyvinylidenefluoride membrane

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sec 61, secretory protein 61; translocon or protein translocating channel

Ser, serine

siRNA, silencing RNA

SMADs, combination of the *Caenorhabditis elegans* protein SMA (Small body size) and *Drosophila* protein MAD (Mothers Against Decapentaplegic)

sPLA2, group IIa secretory phospholipase A2

SRP, signal recognition particle

TG, triacylglycerol

TGFβ, transforming growth factor β

Thr, threonine

TRAM, translocating chain associated membrane protein

Trp, tryptophan

UC, unesterified cholesterol

VLDL, very low density lipoprotein

I INTRODUCTION

Elevated serum levels of apolipoprotein (apo) B are correlated with increased risk of cardiovascular disease (Barter et al. 2006; Liu et al. 2006; Sacks 2006; Sniderman et al. 2006). Noninsulin dependent diabetes mellitus, obesity and the metabolic syndrome are also characterized by an increased hepatic production of apo B containing lipoproteins (Ginsberg et al. 2005; Avramoglu et al. 2006; Ginsberg et al. 2006). Therefore, interventions aimed to reduce the hepatic secretion of apo B containing lipoproteins are of clinical importance. However, the regulation and mechanism of apo B containing lipoprotein biogenesis are complex and poorly understood.

The focus of this thesis is to determine the mechanisms by which the liver assembles apo B with lipids to produce a lipoprotein. In the first part of this study, specific amino acid sequences within apo B are examined to determine if they affect the initial stages of apo B protein entry into the secretory pathway. The second part of this study lays the foundation for determining the contribution of the three distinct synthetic pathways leading to phosphatidylcholine (PC) that is incorporated into apo B containing lipoproteins and the intracellular sites at which these contributions may occur. Therefore, the first part of the introduction gives a general overview of apo B lipoprotein metabolism in the body followed by a more in depth review of the literature encompassing the structure, synthesis, secretion and regulation of apo B. In the second part of this section, a general review of PC synthesis and what is currently known about this lipid's assembly into an apo B containing lipoprotein is presented.

In the final segment of this chapter, based on the literature cited, the thesis objectives are outlined.

I.1 Role of apolipoprotein B in the circulation

Major lipids in the blood are unesterified cholesterol (UC), triacylglycerol (TG), cholesteryl ester (CE), and phospholipids. These lipids are either synthesized by the body or obtained from the diet. In mammals, lipids are transported in the circulation via lipoproteins. Lipoproteins consist of a hydrophobic lipid core of TG and CE surrounded by a monolayer of PL and UC and proteins referred to as apolipoproteins. Apolipoproteins serve to stabilize the macromolecular complex and play major roles in the metabolism of the lipoprotein (e.g. as receptor ligands or control of enzyme activity). Lipoproteins are classified by their buoyant density, summarized in Table I.1 and depicted in Figure I.1.

Table I.1 Physical and chemical characteristics of the major lipoprotein classes
From (Ginsberg et al. 2005) and (Havel and Kane 2001). MM, molecular mass; CM, chylomicron; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

				Lipid (%)		
Lipoprotein	Density (g/ml)	MM (X 10^6 Da)	Diameter (nm)	TG	UC+CE	PL
CM	0.95	400	75-1200	80-95	2-7	3-9
VLDL	0.95-1.006	10-80	30-80	55-80	5-15	10-20
IDL	1.006-1.019	5-10	25-35	20-80	20-40	15-25
LDL	1.019-1.063	2.3	18-25	5-15	40-50	20-25
HDL	1.063-1.21	1.7-3.6	5-12	5-10	15-25	20-30

Major apolipoproteins are described in Table I.2. These proteins are divided into two broad categories: non-exchangeable and exchangeable apolipoproteins (Jonas 2002).

Apo B100 and apo B48, the principle components of chylomicrons, VLDL, IDL, and LDL, are nonexchangeable. These large hydrophobic proteins are assembled and secreted with lipids from intestinal or liver cells and remain bound to the same lipoprotein particle throughout its lifespan in the plasma. In contrast, the exchangeable apolipoproteins (e.g. apo AI) are much smaller than the apo B proteins and are soluble in their delipidated state. These proteins can transfer between lipoproteins and acquire lipids while in the circulation.

Figure I.1 Visual schematic and scanning electron micrograph for comparison of the major lipoprotein classes

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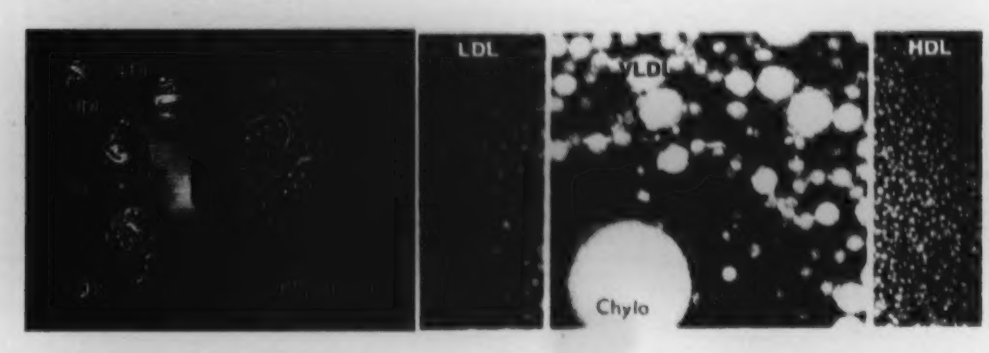


Metabolism of Chylomicrons (CM) and VLDL is shown in Figure I.2 and Figure I.3, respectively. CM are the largest lipoproteins and primarily contain TG and cholesterol from the diet. Apo B48 and microsomal triacylglycerol transfer protein (MTP) are essential for CM assembly (Apo B48 and MTP are discussed in more detail in section I.3 and I.4.3, respectively). CM are released into the lymph where they are then delivered to the blood. CM are acted upon by lipoprotein lipase

(LPL) present on blood vessel walls liberate fatty acids from TG for use by peripheral cells for energy or for storage in adipose tissue. This process results in the formation of chylomicron remnants.

Table I.2 Characteristics of the major apolipoproteins

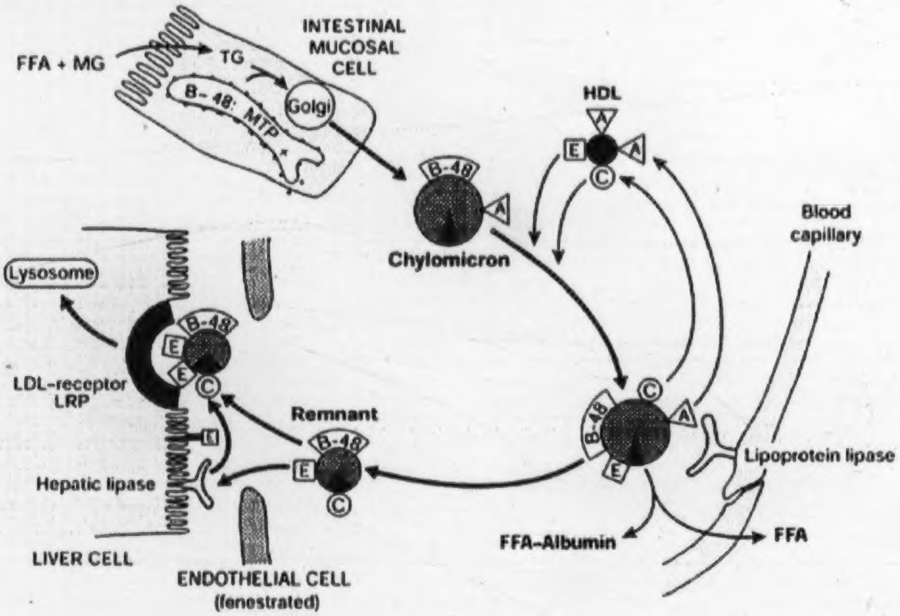
Based on (Ginsberg et al. 2005): LCAT, lecithin cholesterol acyltransferase; CM, chylomicron; LPL, lipoprotein lipase; LRP, LDL-receptor related protein *(Gallagher et al. 2004; Lu et al. 2006); **(Rensen et al. 2005)

Apolipoprotein	MW (X 10^3 Da)	Lipoprotein	Function
Apo AI	28.06	HDL, CM	Structure of HDL; LCAT activator
Apo AII	17.4	HDL, CM	Unknown
Apo AIV	46.465	HDL, CM	Facilitate CM assembly & lipid transport*
Apo AV	39	HDL	Inhibit TG synthesis or enhance lipolysis without effect on apo B secretion**
Apo B48	264	CM	Necessary for assembly and secretion from small intestine
Apo B100	512	VLDL, IDL, LDL	Necessary for assembly and secretion of VLDL from liver; structure of VLDL, IDL, LDL; LDL-receptor ligand
Apo CI	6.63	CM, VLDL, IDL, HDL	Inhibitor of hepatic uptake of CM and VLDL
Apo CII	8.9	CM, VLDL, IDL, HDL	Activator of LPL
Apo CIII	8.8	CM, VLDL, IDL, HDL	Inhibit LPL; Inhibitor of hepatic uptake of CM and VLDL

Apolipoprotein	MM	Lipoprotein	Function
Apo E	34.145	CM, VLDL, IDL, HDL	Ligand for binding LDL-receptor or LRP and proteoglycans

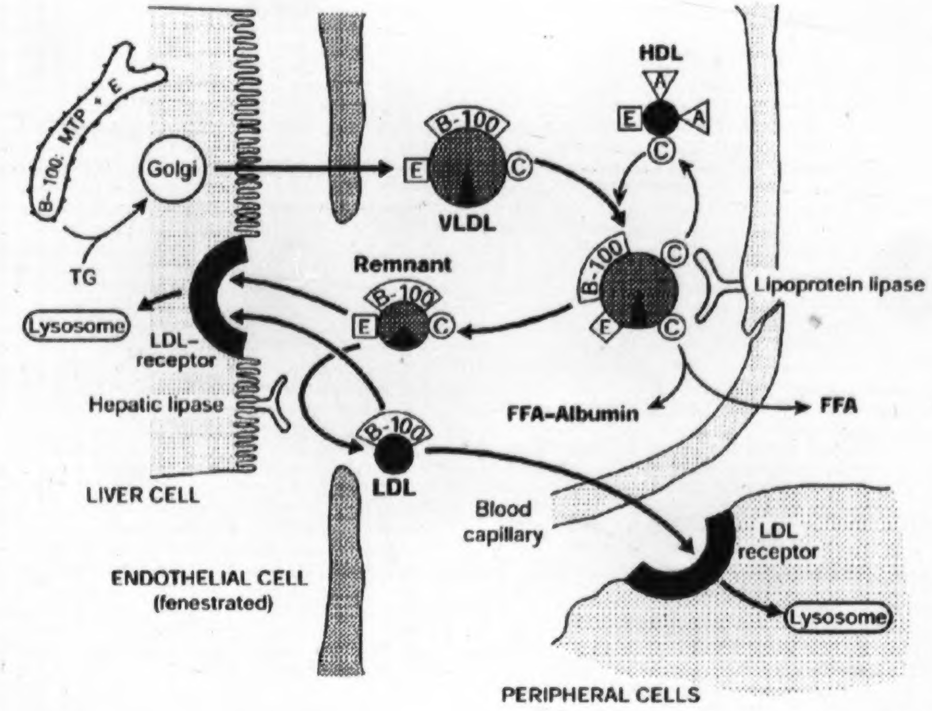
Figure I.2 Chylomicron metabolism

FFA, free fatty acid; MG, monoacylglycerol; MTP, microsomal triacylglycerol transfer protein; B-48, B-100, A, E, C refer to the apolipoproteins listed in Table I.1 and I.2; black semi-circle, LDL-receptor or LRP; image from (Havel and Kane, McGraw-Hill Ryerson ©2001). Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.



Similarly, VLDL serves as the transporter of lipid synthesized by the liver (Figure I.3). VLDL consist of apo B100 (B-100 in figure) and also require MTP for assembly. VLDL are also substrate for TG hydrolysis by lipoprotein lipase, resulting in the formation of VLDL remnants or IDL, which are further catabolized by lipoprotein lipase or hepatic lipase to form cholesterol rich LDL. LDL are taken up by peripheral cells as a source of cholesterol or cleared by the liver by the LDL-receptor.

Figure I.3 VLDL metabolism
See legend of Figure I.2 for details.



I.2 Apolipoprotein B100

I.2.1 Structure of apo B100

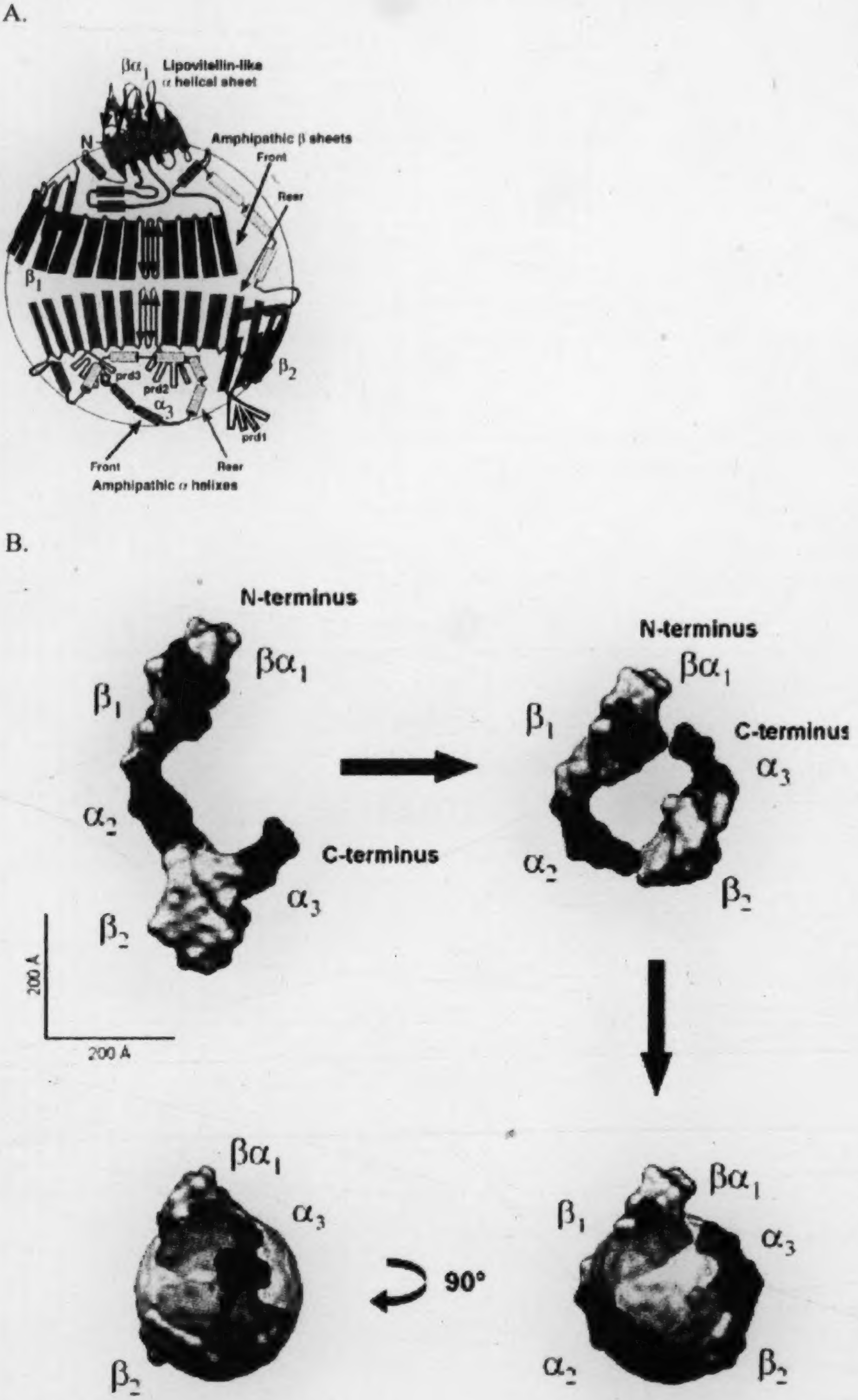
Apo B plays a crucial role in human lipoprotein metabolism. The human apo B gene (*APOB*), located on chromosome 2, produces two forms of apo B, apo B48 and apo B100, named according to a centile scale, collectively referred to as apo B. Apo B48 is identical to the amino terminal 48% of apo B100 and is produced by the small intestine (production of apo B48 is described in section I.3), whereas full-length apo B100 is produced in the liver.

Because of its large size, propensity to form insoluble aggregates in aqueous solutions, and its sensitivity to proteolytic degradation, the primary structure of apo B was not elucidated until the late 1980s (Chen et al. 1986; Yang et al. 1986; Yang et al. 1989). The predicted molecular mass of apo B100 is 513 kDa, and the higher apparent molecular mass of the protein (~550 kDa) found in the circulation is the result of glycosylation at 16 sites in apo B (Yang et al. 1989).

I.2.2 Conformation of apo B100 on a lipoprotein and lipid binding

Apo B is one of three members of the large lipid transport protein superfamily (Smolenaars et al. 2006). Recent models of the three dimensional (3D) conformation of apo B100 on a lipoprotein are displayed in Figure I.4 A. and B. Apo B100 contains hydrophobic lipid-binding regions as well as hydrophilic regions that interact with the aqueous plasma environment. These regions were predicted to be arranged as a series of amphipathic α-helices and β-sheet domains, designated NH_2-βα1-β1-α2-β2-α3-COOH (Segrest et al. 2001; Richardson et al. 2005; Shelness and Ledford 2005; Smolenaars et al. 2006). The amino (N) terminal βα1-domain of ~900 amino acids in length is a globular domain with homology to lipovitellin, an egg yolk lipoprotein and another member of the large lipid transport protein superfamily (Segrest et al. 2001; Smolenaars et al. 2006). This domain initiates lipid binding and primes additional lipid binding of more carboxyl (C) terminal clusters of α helices and β sheets. MTP, the third member of the large lipid transfer superfamily, may bind this region during apo B assembly (section I.4.3). The β domains may be irreversibly associated with the lipid core of the lipoprotein, whereas the α-helical domains, which are similar to those found in the exchangeable apolipoproteins, are thought to exhibit reversible lipid binding thus accommodate for changes in size of the lipoprotein as would occur during assembly and secretion and subsequent catabolism by lipoprotein lipase in the circulation (Wang et al. 2006).

Figure I.4 Proposed structure of apo B100 on a lipoprotein
A. Simplified cartoon predicting pentapartite structure of apo B100 on an LDL particle; prd, proline-rich domain (Richardson et al., The Biophysical Society ©2005). B. Low resolution structure predicted from small angle neutron scattering in combination with advanced shape reconstruction algorithms (Johs et al., The American Society for Biochemistry and Molecular Biology ©2006): top left; extended structure of detergent solubilized apo B100; top right, predicted structure based on arbitrary movement of the carboxyl (C) terminus toward the amino (N) terminus in order to fit a hypothetical model of the spatial arrangement of apo B100 in LDL. Bottom right, model of the LDL particle after superimposition of a 250 Å sphere onto the structural model of apo B100 representing the lipid components. Bottom left: 90° rotation such that the apo B "backbone" is outward, facing the viewer. Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.


A.
$\beta\alpha_1$
Lipovitellin-like
α helical sheet
N
Amphipathic β sheets
Front
Rear
β_1
β_2
prd3
prd2
α_3
prd1
Front
Rear
Amphipathic α helixes
B.
N-terminus
$\beta\alpha_1$
β_1
α_2
C-terminus
α_3
β_2
200 Å
200 Å
N-terminus
$\beta\alpha_1$
β_1
C-terminus
α_3
α_2
β_2
$\beta\alpha_1$
α_3
90°
β_2
$\beta\alpha_1$
β_1
α_3
α_2
β_2

Recently, another 3D model for apo B on an LDL particle was developed based on up-to-date computer modeling and agreement with experimental data available (Krisko and Etchebest 2007). The protein was divided into eight domains and a 3D structure predicted for each of them. Based on the analysis of hydrophobic patches and polar domains, coupled with functional predictions based on the 3D models, precise regions of lipid interaction, LDL receptor binding, and areas of flexibility were defined. The relative organization of the domains reveals a shape quite compatible with the low resolution electron microscopy mapping in which a "book shaped" apo B of 689 Å in length and width between 20 -70 Å was proposed and the C terminus overlaps with the N terminus on the LDL particle.

I.2.3 LDL receptor and proteoglycan binding regions

In addition to its structural role, apo B100 is a ligand for receptor-mediated endocytosis of LDL. Apo B100 contains a LDL-receptor-binding domain that has been localized to the carboxyl-terminal portion of apoB100, specifically; residues 3147-3157 (site A) and 3359–3369 (site B) of the apo B100 primary amino acid sequence (Yang et al. 1986; Yang et al. 1989). Site B is highly conserved among species and most similar to the receptor-binding region in apo E required for chylomicron remnant clearance. Normal binding of LDL to the LDL receptor was shown in transgenic mice expressing apo B100 in which site B was replaced with the equivalent apo E receptor-binding domain (Boren et al. 1998; Tran et al. 1998). Other parts of apo B100 also play an important role in LDL-receptor binding. For example, conformational changes involving interaction between amino acid residues Arg-3500 and Trp-4369 have been demonstrated to be important to induce the structure of apo B100 necessary for binding to the LDL receptor (Boren et al. 1998; Boren et al. 2001).

The atherogenicity of apo B100-containing LDL is linked to their affinity for artery wall proteoglycans (Gustafsson and Boren 2004). Site B, in addition to binding the LDL receptor, has also been identified as the major proteoglycan-binding sequence in apo B100 (Boren et al. 1998). Although this region is not present in apo B48, residues 84–94 were identified as its proteoglycan-binding domain, a site "masked" by the carboxyl terminus in full length apo B (Flood et al. 2002). Site A may become exposed and act cooperatively with site B proteoglycan binding only when plasma LDL phospholipid is modified by group IIa secretory phospholipase A2 (Flood et al. 2004).

I.2.4 Cysteines and covalent modifications

The mature apo B100 protein contains 25 cysteine residues, 16 of which are involved in intramolecular disulfide linkage. Of the eight disulfide bonds, seven occur within the first 1000 residues of apo B (the βα1-domain) and are important for the proper folding of the amino-terminal end of apo B. Proper disulfide bonds in this region are essential for apo B-containing lipoprotein assembly and secretion (Shelness and Thornburg 1996; Tran et al. 1998).

Apo B100 has 19 potential N-glycosylation sites (Asn-X-Ser/Thr), of which 16 asparagine residues are found to be glycosylated (Yang et al. 1989). The carbohydrate moieties were linked to asparagine residues at the following 16 positions: 158, 956, 1341, 1350, 1496, 2752, 2955, 3074, 3197, 3309, 3331, 3384, 3438, 3868, 4210, and 4404. The carbohydrate structures of the N-linked sugar chains of human apo B100 were reported to be high-mannose, hybrid, and mono- and disialylated complex type oligosaccharides (Garner et al. 2001). A seventeenth N-glycosylation site (Asn-2212) has been recently reported, as has more detailed molecular characterization of the glycosyl moieties attached to plasma apo B100 (Harazono et al. 2005). The role of N-glycosylation sites for secretion of apo B100 has been investigated by several laboratories. The results of treating cells with pharmacological agents (e.g. tunicamycin) affecting glycosylation are conflicting (Bell-Quint et al. 1981; Siuta-Mangano et al. 1982; Siuta-Mangano et al. 1982; Liao and Chan 2001). Further, glycosylation sites do not seem to be evoluntionarily conserved amongst species (Smolenaars et al. 2006). However, site directed mutagenesis studies indicated N-linked oligosaccharides at the amino terminus of human apo B100 are important for the assembly and secretion of VLDL (Vukmirica et al. 2002). In addition, glucosamine induced ER stress results in defective N-glycosylation (Qiu et al. 2006). Defective glycosylation leads to conformational changes and reduced secretion of apo B100 from hepatocarcinoma cells (Qiu et al. 2005; Qiu et al. 2006).

Seven of the N-glycans are predicted to occur close to the LDL-receptor binding region of apo B100 and may be important for LDL-receptor interaction; however, the role of the carbohydrate side chains of apo B after secretion into the plasma is not well understood.

Phosphorylation of serine and threonine residues of intracellular apo B has been reported (Davis et al. 1984; Sparks et al. 1988); although the significance of these modifications has not been determined. Palmitolyation of free cysteine might have impact on lipidation, intracellular localization or retention and/or partitioning of the apo B containing lipoprotein between soluble and membrane associated forms (Zhao et al. 2000; Vukmirica et al. 2003; Vilas and Berthiaume 2004).

1.3 Apolipoprotein B48

A unique mRNA editing process enables the *APOB* gene to make the two structurally related but discrete isoforms from the same 14.3 kb mRNA (Davidson and Shelness 2000; Anant and Davidson 2001; Chen et al. 2007). Briefly, editing of apo B mRNA occurs in all mammalian small intestinal cells and in liver cells of some animal species including rodents. The process involves an RNA-specific cytidine deamination of the cytosine molecule at base 6666 of the mRNA. Deamination creates a uracil, resulting in the glutamine 2153 codon (CAA) being converted to a termination codon (UAA). This editing process is mediated by an editosome of 27 S consisting of the cytidine deaminase apo B mRNA editing enzyme catalytic complex 1 (apobec-1) and ACF (apobec-1 complementation factor), a novel RNA-binding protein that serves as the RNA recognition component of the editing enzyme. A minimal requisite sequence region size of ~30 nucleotides flanking cytidine 6666 has been identified. This region includes a conserved 11 nt sequence located 4-6 nt downstream of the cytidine that is essential for *in vitro* mRNA editing to occur. Other auxiliary proteins involved include CUG binding protein 2 (CUGBP2), glycine-arginine-tyrosine-rich RNA binding protein (GRY-RBP), heterogeneous nuclear ribonucleoprotein-C1 (hnRNP-C1), KH-type splicing regulatory protein (KSRP), apobec-1 binding protein (ABBP)1 and 2, and Bcl-2-associated athanogene 4 (BAG4) and auxiliary protein 240 (AUX240). These proteins have been identified by using detection assays that primarily reflect their ability to interact with apobec-1, ACF, or apo B RNA but do not establish if these factors play a role *in vivo*.

Apo B mRNA editing activity and expression is responsive to a number of stimuli such as thyroid hormone, insulin, nutritional status and developmental stage. For example, recent work (Chen et al. 2007) demonstrates that the dramatic developmental up-regulation of apo B mRNA editing in mouse small intestine begins with down-regulation of inhibitory CUGBP2 expression, followed by up-regulation of apobec-1 and ACF, which is followed by down-regulation of the inhibitory components GRY-RBP and hnRNP-C1. The expression of other editing components is relatively unaltered. Further investigation using silencing RNA (siRNA) mediated gene specific knock down of CUGBP2, GRY-RBP, hnRNP-C1, KSRP, ABBP1, BAG4 and ABBP2 all resulted in an increased level of editing in Caco-2 cells, an intestinal cell line model, indicating that these components directly or indirectly play an inhibitory role in the editing process.

The functional significance of apo B48 is not fully understood. Apo B48 may be a more efficient vehicle for fat absorption under specific conditions (such as limited food or fat availability). Thus mRNA editing might have conferred an evolutionary advantage (Kendrick et al. 2001). However, apo B48 can bind arterial walls, therefore can be proatherogenic (see section 1.2.3). Apo B48 overproduction has also been observed in disease such as insulin resistance, (Avramoglu et al. 2006).

I.4 Synthesis and secretion of apo B

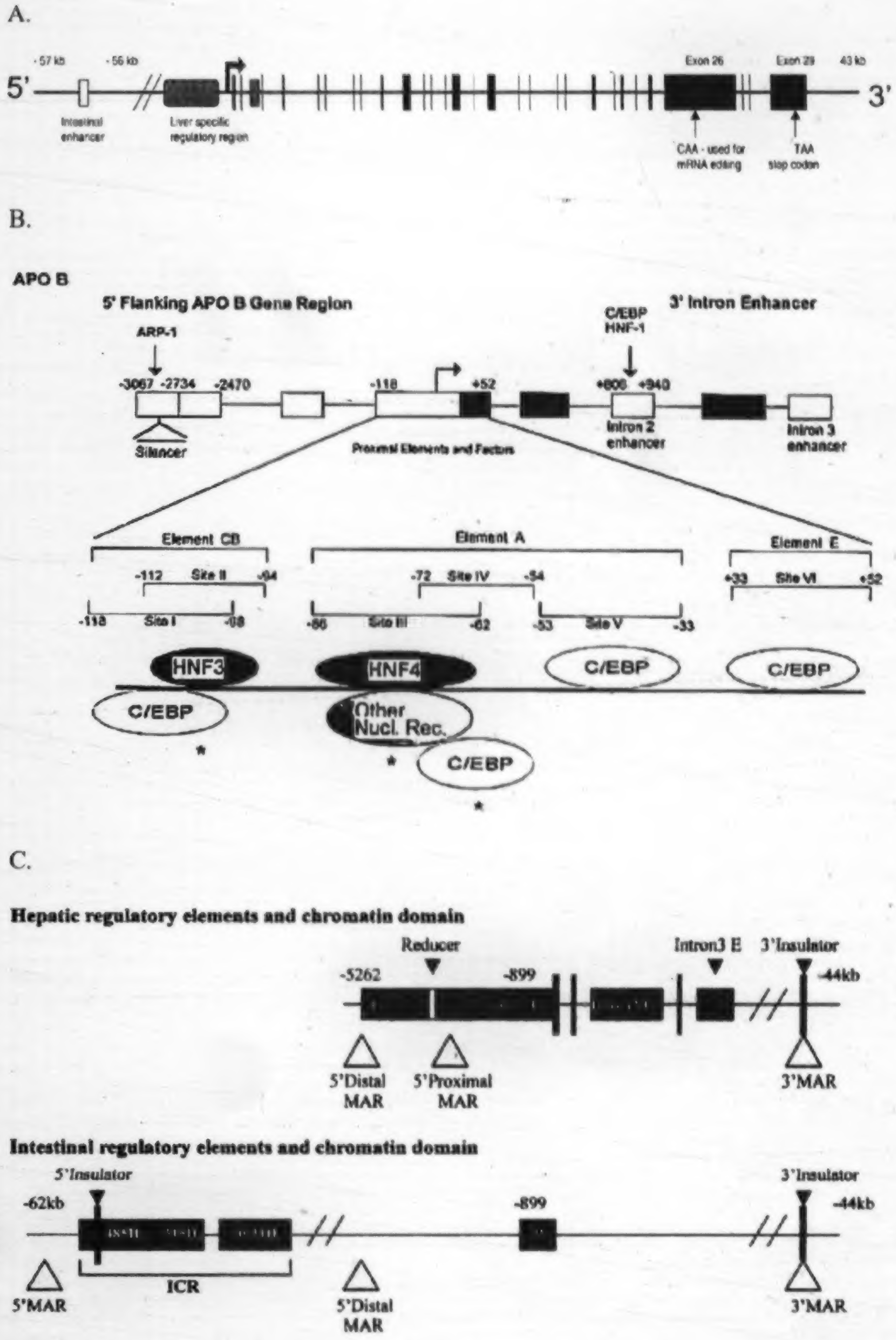
I.4.1 Gene expression

Apo B gene expression is reviewed by (Zannis et al. 2001; Zannis et al. 2001; Wang et al. 2003). The *APOB* gene is 43 kb in length and located on the short arm of human chromosome 2. The *APOB* gene consists of 29 exons, of which exon 26, at 7572 bp, is the largest and encodes more than one-half of the full-length protein. *APOB* gene expression is largely confined to the liver and intestine although low levels are detectable in other tissue such as aortic endothelial cells, the heart, and the placenta (Sivaram et al. 1996; Boren et al. 1998; Bjorkegren et al. 2001; Madsen et al. 2004). Figure I.5 depicts the structure of the *APOB* gene (A.), localization of some transcription binding factors, specific control regions (B.) and the organization of the chromatin (C.) discussed in more detail below.

Transcription of *APOB* is regulated by a proximal promoter (-150 to -124) which binds hepatic nuclear factors 3 and 4 (HNF-3, HNF-4) and CCAT/enhancer binding protein (C/EBP). The promoter works in combination with either liver-specific regulatory regions (liver control regions, LCR) or intestinal-specific regulatory regions (intestinal control regions, ICR) to confer tissue specificity. The LCR is located within nucleotides -899 to -5262 and requires an enhancer located in intron 2. The ICR is located 56 kb upstream of the promoter and includes three important regions. A 315-bp region sufficient for intestinal expression alone binds intestine-enriched HNF-4, HNF-3β and C/EBPβ. A 485 bp enhancer positioned immediately upstream of the 315 bp region can also confer intestine-specific expression. This enhancer binds HNF-4/ARP-1 as well as SMADs, transcription factors that may convey transforming growth factor β (TGFβ) signaling and enhance transcription of the *APOB* gene in intestinal cells (Singh et al 2002). [The term SMAD is derived from the founding members of this family, the Drosophila protein MAD (Mothers Against Decapentaplegic) and the Caenorhabditis elegans protein SMA (Small body size)]. Finally, a 1031 bp regulator 1.2 kb downstream of the 315 bp has both enhancer and inhibiting properties. This region may bind intestine enriched C/EBPβ and GATA-4 transcription factors.

Figure I.5 Structure of apo B gene and location of tissue specific enhancer regions

A. Gene structure (Hooper et al., Informa ©2005): bent arrow, promoter region; black boxes, exons. B. Position of binding proteins in the LCR from (Zannis et al., Lippincott Williams and Wilkins ©2001). C. Closer examination of liver and intestinal specific enhancer regions including matrix associated regions (MAR, upward pointing light arrowhead) and insulators (vertical lines demarked with black downward pointing arrowhead marked insulator) (Wang et al., Elsevier ©2003): L, liver specific enhancers (grey boxes demarked with an E); P, promoter (dark gey box demarked with a P); ICR, intestinal control region (grey boxes demarked with an E and numbers).. Note that the silencer/reducer (apolipoprotein AI apolipoprotein regulatory factor 1 (ARP-1) binding region in the LCR upstream of the promoter, light grey box, indicated by black downward pointing arrowhead demarked reducer) appears to exert its effect only *in vitro* (Zannis et al. 2001). Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.


A.
- 57 kb
- 56 kb
Exon 26
Exon 29
43 kb
5'
3'
Intestinal enhancer
Liver specific regulatory region
CAA - used for mRNA editing
TAA stop codon
B.
APO B
5' Flanking APO B Gene Region
3' Intron Enhancer
ARP-1
C/EBP
HNF-1
-3067
-2734
-2470
-118
+52
+806
+940
Silencer
Proximal Elements and Factors
Intron 2 enhancer
Intron 3 enhancer
Element CB
Element A
Element E
-112
Site II
-94
-72
Site IV
-54
+33
Site VI
+52
-118
Site I
-98
-86
Site III
-62
-53
Site V
-33
HNF3
HNF4
C/EBP
C/EBP
C/EBP
Other Nucl. Rec.
C/EBP
*
*
*
C.
Hepatic regulatory elements and chromatin domain
Reducer
Intron3 E
3'Insulator
-5262
-899
-44kb
5'Distal MAR
5'Proximal MAR
3'MAR
Intestinal regulatory elements and chromatin domain
5'Insulator
3'Insulator
-62kb
-899
-44kb
ICR
5'MAR
5'Distal MAR
3'MAR

I.4.2 Apo B synthesis and translocation into the ER lumen

Protein biogenesis in the secretory pathway is reviewed (Alberts et al. 2002); a simplified animation of the early steps of protein synthesis and targeting to the ER is available at http://www.rockefeller.edu/pubinfo/proteintarget.html; see also (Reithmeir 1996; Schnell and Hebert 2003; White and von Heijne 2004; Osborne et al. 2005) and references cited within. The process involves targeting of nascent peptides to the membrane of the endoplasmic reticulum (ER), translocation into the lumen of the ER, folding and modification (e.g. glycosylation), as well as quality control in which malformed proteins may be translocated back into the cytoplasm and degraded by the proteasome. Integral membrane proteins must also be inserted into the membrane before being sorted to their final location. In mammals, these processes can occur co-translationally, i.e., concomitant with peptide synthesis.

Nascent peptides destined for the ER contain N terminal signal sequences that are recognized by the signal recognition particle (SRP). SRP binds the signal sequence and arrests further translation of the peptide until the SRP-ribosome-nascent peptide complex reaches the ER. The complex is in turn is recognized via SRP by the SRP receptor in the ER membrane. This receptor is coupled to an oligomeric membrane complex, the translocon, which mediates the translocation or movement of peptides across or into the membrane. In the ER, the mammalian core components of the translocon are the Sec61α, Sec61β and Sec61γ subunits and translocating chain associated membrane protein, TRAM, a 35 kDa protein which promotes membrane integration of transmembrane proteins. SRP and the SRP receptor act to expose the translocon binding site on the ribosome to result in the release of the SRP unit and engagement of the ribosome-nascent peptide with the translocon (Halic et al. 2006). The signal sequence on the nascent peptide is cleaved by signal peptidase and translation of the peptide is resumed. The ribosome usually forms a tight seal with the Sec61 complex. For membrane proteins with cytosolic domains, the ribosome may tilt to allow cytosolic exposure of the nascent peptide during protein synthesis (Alder and Johnson 2004); or, based on cyro-electron microscopy studies, there may be a pre-existent 15 Å gap between the ribosome and the translocon that may permit egress of some nascent peptide chains into the cytosol (Menetret et al. 2000; Beckmann et al. 2001). The lumenal ER resident protein immunoglobulin heavy chain binding protein (BiP) or glucose response protein (GRP 78) associates with the translocon and appears to maintain the permeability barrier of the ER (Alder and Johnson 2004; Alder et al. 2005) as well as acts as a molecular ratchet, which prevents the nascent peptide chain from sliding back into the cytosol (Eichler and Irihimovitch 2003; Osborne et al. 2005).

Like other secreted proteins, apo B is synthesized on attached ribosomes of the ER. The amino terminus of apo B includes a polymorphic signal peptide of either 24 or 27 amino acids that is co-translationally cleaved to produce a mature protein of either full length apo B100 or apo B48 (Yang et al. 1986; Yang et al. 1989). However, while most secretory proteins undergo efficient co-translational translocation into the lumen of the ER, evidence has accumulated that indicates that the translocation of apo B may not be efficient. Apo B lacks transmembrane domains (Yang et al. 1989). Instead, the peptide may possess pause transfer sequences (PTS) that confer a delay, or a transient pause, during translocation into the ER so that the peptide is transiently exposed to the cytosol before resuming translocation into the ER lumen (Chuck et al. 1990; Chuck and Lingappa 1992; Chuck and Lingappa 1992; Chuck and Lingappa 1993), although this phenomenon has been questioned by other investigators (Pease et al. 1991; Shelness et al. 1994; Pease et al. 1995; Liang et al. 1998). One of the objectives of this thesis is to determine if PTS can function in cell culture.

I.4.3 Microsomal triacylglycerol transfer protein

MTP, likely the primordial member of the large lipid transfer protein superfamily (Smolenaars et al. 2006), plays an essential role in the lipidation and secretion of apo B in mammals (Shelness and Sellers 2001; Hussain et al. 2003; Shelness and Ledford 2005). MTP is a heterodimer consisting of a primarily liver and intestinal specific 97 kDa subunit that performs the transfer of TG, CE and phospholipid between phospholipid surfaces, and a ubiquitous 58 kDa subunit, protein disulfide isomerase (PDI), which may stabilize and prevent aggregation of the MTP component as well as help retain this subunit in the ER compartment (Lamberg et al. 1996), although MTP has been detected in the Golgi (Levy et al. 2002; Swift et al. 2003). The importance of MTP is emphasized by the disease abetalipoproteinemia (OMIM #200100) in which mutations of the *MTP* gene, that abrogate or reduce its expression or its function, result in fat malabsorption, failure to thrive, low plasma cholesterol and TG, and almost complete absence of apo B containing lipoproteins in the plasma (Sharp et al. 1994; Kane and Havel 2001). Further support for the involvement of MTP in apo B containing lipoprotein formation is observed when apo

B and MTP are co-expressed in non-lipoprotein producing cell lines; in the absence of MTP, apo B is not secreted (Gordon et al. 1994; Leiper et al. 1994).

MTP may be essential for the translocation of apo B by acting as a chaperone and/or for lipidation for assembly and secretion (discussed in more detail in the next section).

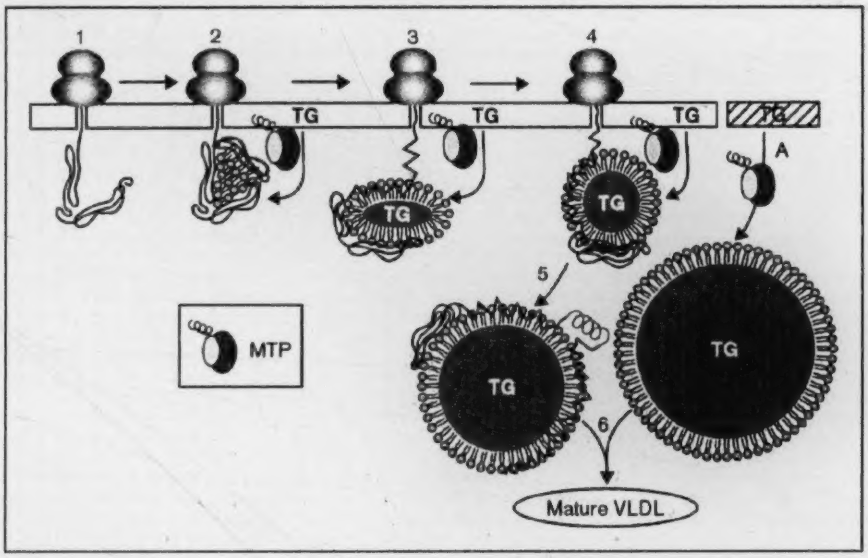
I.4.4 Current models of lipoprotein assembly

Apo B containing lipoprotein assembly is still poorly understood, particularly with respect to how the bulk of lipid is added and the site where that addition occurs. Lipoprotein synthesis may involve at least two steps: co-translational lipidation results in the formation of a primordial lipid particle that may then fuse with or acquire lipid from a lumenal lipid droplet (Figure I.6) (Alexander et al. 1976; Rustaeus et al. 1999; Cartwright and Higgins 2001).

Apo B interacts with several molecular chaperones, including GRP94, ERp72, BiP, calreticulin and cyclophilin B (Linnik and Herscovitz 1998). MTP may act as a chaperone and/or may initiate lipid binding to apo B as it is synthesized by forming a lipid binding pocket with the N terminal portion of apo B (Dashti et al. 2002). A more recent model relies on sequences within the first 1000 amino acids of apo B to form this pocket (step 1 Figure I.6) (Manchekar et al. 2004; Richardson et al. 2005). This pocket is filled with mainly phospholipid (step 2 Figure I.6), and is then converted to a nascent core-containing emulsion particle as the translation of apo B continues (step 3 and 4 Figure I.6). Alternately, apo B may directly interact with the ER membrane to nucleate oil droplet formation that desorbs from the ER as an emulsion (Shelness and Ledford 2005; Ledford et al. 2006). In a second stage, following translation (step 5 Figure I.6), apo B acquires most of its neutral lipids (TG and CE), perhaps by fusion with a neutral lipid droplet (step 6 Figure I.6). This intralumenal lipid is accreted in the secretory pathway via MTP (step A Figure I.6) but the final step, the formation of mature VLDL appears to be MTP independent (Raabe et al. 1999; Wang et al. 1999; Kulinski et al. 2002).

Figure I.6 Two step model of lipoprotein assembly.

Two step model of apo B lipoprotein assembly is described in the text; image from (Shelness and Sellers, Lippincott Williams and Wilkons ©2001). Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.



An alternate model of lipoprotein assembly suggests lipid may be added sequentially to achieve the full complement of a mature VLDL molecule (Janero and Lane 1983; Bostrom et al. 1986; Bostrom et al. 1988; Boren et al. 1990).

Either model could occur in the ER (Rusinol et al. 1993; Kulinski et al. 2002; Yamaguchi et al. 2003), or during transport and/or within the Golgi (Janero and Lane

1983; Bamberger and Lane 1988; Bostrom et al. 1988; Stillemark et al. 2000; Tran et al. 2002; Gusarova et al. 2003).

Although the source of TG for VLDL assembly is still not clear, the origin of the CE that is packaged into the VLDL core has been clarified (Shelness and Sellers 2001). Two intracellular cholesterol-esterifying enzymes, acyl coenzyme A:cholesterol acyltransferase (ACAT)-1 and -2, have been identified (Chang et al. 1993; Anderson et al. 1998; Cases et al. 1998; Cases et al. 1998; Oelkers et al. 1998). ACAT-2 is considered to be the major enzyme responsible for hepatic CE formation in mice and nonhuman primates while ACAT-1 is responsible for cholesterol esterification in adult human liver (Chang et al. 2000; Lee et al. 2000).

PC is the major phospholipid in lipoproteins secreted by the liver (Jonas 2002). PC synthesis is discussed in more detail in Section I.6. However, the intracellular site(s) whereby the PC derived from specific phosholipid pools is (are) added to the lipoprotein has not been determined. One of the objectives of this thesis is to determine the contribution of the different PC biosynthetic pathways to VLDL PC and the intracellular locations in which this occurs.

I.4.5 Cellular trafficking and secretion of apo B containing lipoproteins

Classical secretory vesicles for transporting proteins are 50-80 nm in diameter yet hepatic apo B containing lipoproteins can be up to 200 nm in diameter. Recent *in vitro* evidence suggests lipid poor apo B lipoproteins depart the ER in specific coatamer protein complex II (COPII) vesicles that exclude other secretory proteins (Gusarova et al. 2003; Brodsky et al. 2004); COPII vesicle assembly is reviewed in (Gurkan et al. 2006; Sato and Nakano 2007). However, these investigators failed to show that these vesicles were competent to fuse with the Golgi. Sar 1a and 1b, two guanidine exchange proteins that stimulate coatamer formation and vesicle budding from the ER, do appear to be important for lipoprotein secretion since expression of dominant negative forms of Sar1 protein prevent apo B from being secreted. Liver and intestine may use distinct isoforms of this protein since mutations in SARA2 gene (that encodes Sar1b) give rise to Anderson's disease (OMIM#607689), Chylomicron Retention Disease (CMRD,OMIM#246700), or CMRD-Marinesco Sjogren (OMIM#607692), in which chylomicrons accumulate in intestinal cells, however, VLDL secretion from the liver is unaffected (Kane and Havel 2001; Jones et al. 2003). Recent work in Balch's laboratory also indicates that the COPII apparatus may accommodate large cargo like apo B containing lipoproteins without so much size-constraint (Gurkan et al. 2006).

Apo B containing lipoproteins may undergo further remodeling and modification in post-ER compartments before they are eventually secreted.

I.4.6 Post-translational degradation

Much of apo B protein that is synthesized is degraded within the hepatocyte (Borchardt and Davis 1987; Yao et al. 1997; Fisher and Ginsberg 2002). The degradation of newly synthesized apo B is mediated primarily by the ubiquitin-proteasome pathway in the cytosol. If apo B does not acquire sufficient lipid, it is degraded by the ubiquitin-proteasome pathway while remaining associated with the translocon and attached to the ribosome (Pariyarath et al. 2001). Degradation of apo B is mediated by chaperones heat shock proteins (Hsp)-70 and -90 (Fisher et al. 1997). Apo B then becomes covalently modified by conjugation to ubiquitin. ER associated Gp78, an E3 ligase, coordinates the covalent binding of ubiquitin to apo B (Liang et al. 2003). Calnexin (CXN) protects apo B from proteasomal degradation; if the interaction between CXN and apo B is inhibited then more apo B undergoes ubiquitinylation and proteasomal degradation (Chen et al. 1998). In contrast, ferritin, a cytosolic iron-storage protein, has been found to bind apo B in HepG2 (Rashid et al. 2002). Overexpressing ferritin results in increased proteasomal degradation of apo B, thus ferritin may behave like chaperones Hsp 70 or 90 under oxidative stress conditions (Rashid et al. 2002; Hevi and Chuck 2003).

Apo B can also be degraded within the lumen of the ER. An ER-localized protein, ER-60, was found to be associated with apo B in a human hepatoma cell line and appears to be involved in apo B degradation through a non-proteasomal pathway (Adeli et al. 1997). However, it is currently unknown whether ER-60 acts directly upon apo B as a protease or if it is involved in targeting apo B for degradation (Qiu et al. 2004; Hooper et al. 2005).

The LDL receptor also has a role in apo B degradation. In the presence of the receptor, an increased proportion of apo B undergoes degradation (Twisk et al. 2000; Gillian-Daniel et al. 2002). The LDL receptor can bind pre-secretory apo B within the ER/Golgi and target it for degradation, or "recapture" newly secreted VLDL, preventing its release into the circulation. After re-uptake, VLDL particles are internalized and delivered to lysosomes for digestion by proteases. Re-uptake of VLDL might be modulated indirectly by proprotein convertase subtilisin-like kexin type 9 (PCSK9), a newly discovered serine protease that may catabolize LDL receptors in liver and thereby control the number of receptors available for clearance of plasma LDL and VLDL re-uptake (Lambert et al. 2006; Horton et al. 2007).

Post-ER, pre-secretory degradation of apo B has also been observed (Fisher et al. 2001) in a pathway stimulated by n-3 fatty acids which increase intracellular lipid peroxidation products, lipid peroxidation, and oxidative stress mechanisms in primary cultured hepatocytes. As a result, apo B secretion is inhibited and degradation of apo B is promoted, a process that can be prevented by the addition of anti-oxidants like vitamin E (Pan et al. 2004). The effect of n-3 fatty acids might be attributable to failure to complete the second step of lipoprotein assembly during which the bulk of TG is enriched with n-3 fatty acid side chains obtained from phospholipid remodeling (Tran et al. 2006). Phospholipid transfer protein (PLTP), a key participant in the transport of hydrophobic molecules within the circulation, may deliver vitamin E to liver cells and thus prevent this post-ER degradative event (Jiang et al. 2005).

A role for cytosolic lipid droplets in apo B degradation has been also been proposed (Ohsaki et al. 2006). Cytosolic lipid droplets are abundant in liver cells. It is generally believed lipid droplets store TG and may provide this lipid via a hydrolysis/re-esterification pathway for VLDL assembly (discussed in Section I.4.4) (Wiggins and Gibbons 1996; Salter et al. 1998; Gibbons et al. 2004). Recently it was determined that cytosolic droplets may also provide a surface for apo B that has been dislocated into the cytoplasm and ubiquinated in Huh7 hepatoma cells. The apo B forms crescents around these lipid droplets that displace their normal coat proteins. It is thought that in this way, toxic insoluble aggregates of apo B do not occur.

Mechanisms of degradation involving both proteasome and autophagy have been proposed (Ohsaki et al. 2006).

I.5 Regulation of apolipoprotein B assembly and secretion

I.5.1 Gene Expression

Apart from the fact that apo B gene expression is tissue-specific and developmentally regulated (Demmer et al. 1986), a number of investigations have demonstrated that apo B gene expression is constitutive under a wide range of conditions that influence its secretion (Pullinger et al. 1989; Sorci-Thomas et al. 1989; Moberly et al. 1990). Therefore, while some studies indicate that steady state levels of apo B mRNA can change, albeit of limited magnitude (<2-3 fold) (Hennessy et al. 1992; Sparks et al. 1992; Zhang et al. 1993; Wu et al. 1994; Dashti et al. 1996; Tachibana et al. 2005; Perez et al. 2006; Sparks et al. 2006), apo B secretion is largely controlled by post-transcriptional and post-translational mechanisms.

I.5.2 Protein synthesis and translocation into the ER lumen

Apo B production can be affected at the level of protein synthesis by insulin (reduced) or thyroid hormone (increased) (Adeli and Sinkevitch 1990; Sparks and Sparks 1990; Adeli and Theriault 1992; Theriault et al. 1992). Specifically, with respect to the effects of insulin, translation of apo B may be regulated by structural properties of the 5' and 3' untranslated regions (UTRs) of the transcript as well as the binding of an 110 kDa insulin sensitive trans-acting factor (Pontrelli et al. 2004; Sidiropoulos et al. 2005).

The phospholipid content of the ER membrane may influence the ability of the apo B peptide to translocate into the ER lumen. Monomethylethanolamine (MME) feeding of primary hepatocytes or hepatoma cells results in enrichment of the ER membrane in phosphatidylmonomethylethanolamine (PMME). PMME specifically inhibits translocation of apo B resulting in a reduction in secretion of VLDL and enhanced degradation (Vance 1991; Rusinol et al. 1993; Rusinol and Vance 1995; Rusinol et al. 1996; Rusinol et al. 1998). This effect may be mediated by failure to restart translocation after encountering PTS (Rusinol et al. 1998).

Concurrent inhibition of proteasomal degradation and MTP can also arrest translocation of the apo B peptide and subsequently inhibit apo B synthesis in HepG2 cells (Mitchell et al. 1998; Pan et al. 2000). Thus MTP may promote translocation through lipidation of the apo B emerging from the translocon into the ER lumen. Accumulation of the peptide in the translocon is thought to affect peptide elongation although the mechanism involved has yet to be elucidated (Mitchell et al. 1998; Pan et al. 2000).

1.8.3 Post-translational regulation

Most studies in cultured liver cells and hepatoma cell lines indicate that a significant proportion of newly synthesized apo B100 is degraded before secretion; if hepatic lipids are unavailable for assembly into VLDL, apo B can be degraded at the ER by the proteasome or distal to the ER in a mechanism yet to be fully established (discussed in Section 1.6.3) (Boström et al. 1986; Borchardt and Davis 1987; Pullinger et al. 1989; Dixon et al. 1991; White et al. 1992; Cartwright and Higgins 1996). The type of fatty acid available may affect whether apo B is secreted or degraded [e.g. oleate stimulates secretion (Bostrom et al. 1988) while n-3 fatty acids promote degradation (Wang et al. 1993), Section I.4.5]. Further, how the fatty acid is delivered to the liver (i.e., conjugated with bovine serum albumin or as an emulsion) may also affect whether apo B is secreted or degraded (Zhang et al. 2004).

Sequences within apo B may dictate whether it is secreted or degraded: specifically, it was found that β sheet regions of apo B were required for efficient translocation, susceptibility to proteasomal degradation, and lipid responsiveness of apo B, although these experiments could not absolutely preclude the involvement of PTS (Liang et al. 1998). In another study, structural elements within the β1 domain of human apo B was found to mediate rapid intracellular degradation by promoting cytosolic exposure of the apo B peptide (Lapierre et al. 2004).

Altered levels of MTP activity and expression modulate lipoprotein production [reviewed in (Gordon and Jamil 2000)]. Indeed, changes in MTP expression or activity have been linked to altered plasma TG levels and VLDL secretion. For example, pharmacological inhibition of MTP leads to a dose dependent decrease of secretion of apo B-containing lipoproteins by hepatoma cells, rabbits, rats, and humans (Benoist et al. 1996; Haghpassand et al. 1996; Jamil et al. 1996; Jamil et al. 1998; Wetterau et al. 1998; Chandler et al. 2003). Heterozygous MTP-deficient mice have reduced rates of apo B secretion, and plasma apo B levels are 70% of those in wild type mice (Raabe et al., 1998). It appears that the concentration of MTP within the ER rather than the MTP:apoB ratio is the critical determinant of lipoprotein secretion (Leung et al. 2000). Overexpression of MTP by recombinant adenoviral infection in mice leads to increased plasma TG and apo B secretion (Liao et al. 1999; Tietge et al. 1999).

Acute insulin treatment acutely inhibits MTP expression but since the half life of MTP is 4.4 days, changes in MTP gene expression, resulting in changes in protein level and activity, would appear to be more related to a chronic adaptation process rather than a mechanism for acute regulation of lipoprotein production (Lin et al. 1995). MTP gene expression has been under intensive study. More recent work links forkhead transcription factor Foxa2 and perioxisome proliferator agonist γ co-activator 1β (PGC-1β) to increased MTP gene expression (Wolfrum and Stoffel 2006). Foxa2 may be subject to inactivation by phosphophorylation by insulin dependent PI-3 kinase-Protein kinase B/AKT pathway (Koo and Montminy 2006). PGC-1 was also found to co-activate perioxisome proliferator receptor α/retinoic acid proliferator receptor α complex (PPARα/RXRα) which competes with chicken ovalbumin transcription factor II (COUP-TII) for direct binding repeat 1 (DR-1) located in the MTP promoter (Spann et al. 2006). The latter factor inhibits, while the former stimulates, MTP gene expression. Other factors such as sterol depletion (Sato et al. 1999); bile acid (Hirokane et al. 2004), bitter melon (Nerurkar et al. 2005), ethanol (Lin et al. 1997), and garlic (Lin et al. 2002) inhibit- while oleate (Qiu et al. 2005) stimulates- MTP expression. MTP gene expression may also be under the control the MAP kinase cascade (Au et al. 2003): cellular MAPK erk stimulates and MAPKp38 inhibits, thereby playing a counterbalancing roles in regulating the MTP gene transcription, independent of PI-3 kinase.

Finally, insulin may mediate its effect on post-ER degradation via PI-3-kinase or possibly MAP kinase pathways that results in up regulation of the LDL receptor and as such, promotes re-uptake of newly secreted VLDL [reviewed in (Avramoglu and Adeli 2004)].

I.6 Phospholipids and apo B

I.6.1 Overview of phospholipid synthesis

The most abundant phospholipid in eukaryotic cell membranes is PC which makes up 40–50% of total phospholipids. Other phospholipids, such as phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM) constitute 15-25%, 2-10%, 10-15% and 5-10% total lipid respectively [reviewed in (Vance and Steenbergen 2005)]. The phospholipid composition of cellular components such as the ER and Golgi of rat liver is given in Table I.3. In apo B containing lipoproteins secreted by the liver, phospholipid constitutes 10-20% of the total lipid (Table I.1) and PC comprises 60-74% of that phospholipid (Jonas 2002).

Phospholipid biosynthesis is summarized in Figure I.7, and discussed below. More detailed reviews of phospholipid synthesis are given in (Vance 1998; Vance 2002; Vance and Vance 2004; Vance and Vance 2005)

I.6.2 Phosphatidylcholine biosynthesis

In all nucleated mammalian cells, PC is made by the CDP choline pathway (Vance 2002). The liver has an additional pathway for PC synthesis, the PE methylation pathway. This pathway contributes 30%–40% of liver PC but has very low activity in other cells; see references cited in (Vance and Vance 2004).

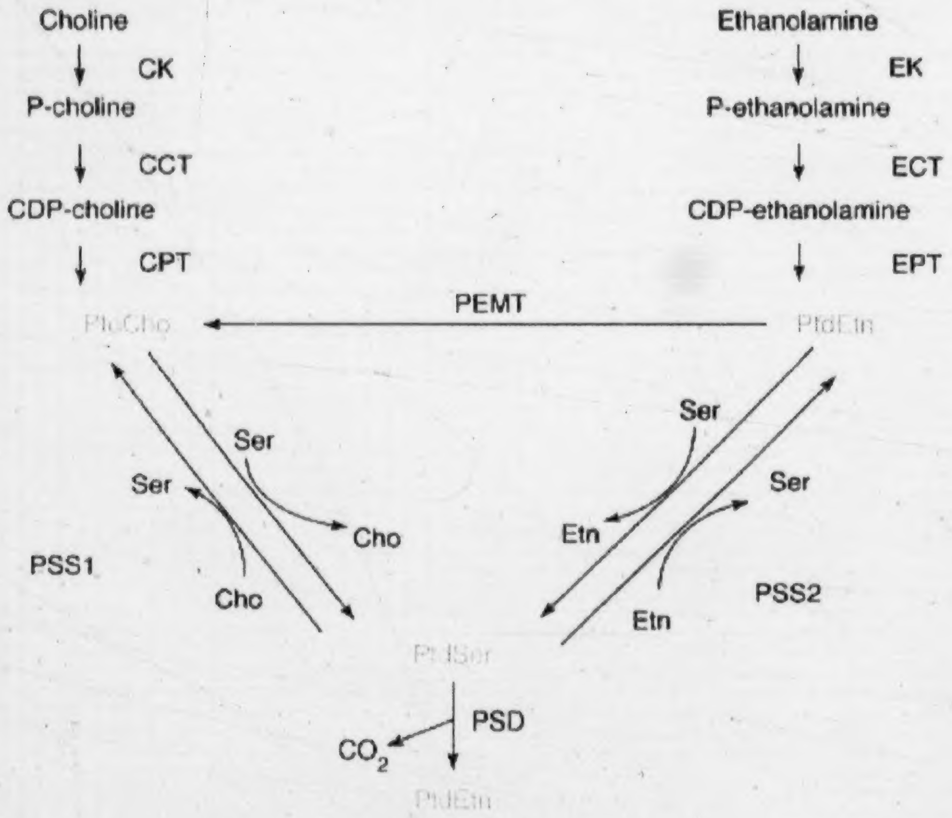
Table I.3 Lipid composition of subcellular organelles from rat liver

Values for individual lipids are percentage of total phospholipid phosphorous; LPC, lysophosphatidylcholine; CL; cardiolipin; *, umole phosphorous/mg protein. From (Colbeau et al., Elsevier ©1971). Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.

Phospholipid	ER		mitochondria		nucleus	Golgi	lysosome	Cell membrane
	rough	smooth	inner	outer				
LPC	2.9	2.9	0.6	-	-	5.9	2.9	1.8
SM	2.4	6.3	2.0	2.2	6.3	12.3	16.0	23.1
PC	59.6	54.4	40.5	49.4	52.1	45.3	41.9	43.1
PI	10.1	8.0	1.7	9.2	4.1	8.7	5.9	6.5
PS	3.5	3.9	1	1	5.6	4.2	-	3.7
PE	20.0	22.0	38.8	34.9	25.1	17.0	20.5	20.5
CL	1.2	2.4	17.0	4.2		-	-	-
PL:protein*	0.33	0.47	0.34	0.46	-	-	0.21	0.37

Figure I.7 Biosynthesis of phospholipids

CK, choline/ethanolamine kinase; CCT, CTP:phosphocholine cytidylyltransferase; CPT, choline phosphotransferase; EK, ethanolamine kinase; ECT, CTP:ethanolamine cytidylyltransferase; EPT, ethanolamine phosphotransferase; PEMT, phosphatidylethanoline methyltransferase; PSS1, phosphatidylserine synthase 1; PSS2, phosphatidylserine synthase 2; PSD, phosphatidylserine decarboxylase; Ser, serine; Etn, ethanolamine; Cho, choline; P-choline, phosphocholine; P-ethanolamine, phosphoethanolamine. From (Vance, Elsevier ©1998). Copied under licence from Access Copyright. Except as permitted by Law, further reproduction is prohibited.



I.6.2.1 CDP-choline or Kennedy pathway

Choline is an essential nutrient needed as a component of PC for the structural integrity and signaling functions of cell membranes; for normal cholinergic neurotransmission as acetylcholine; as the major source of methyl groups for methionine biosynthesis when oxidized to form betaine and for lipid export from liver as a lipoprotein. Choline is rapidly transported into liver cells by two types of transporter: a high affinity sodium dependent transporter and a low affinity sodium independent transporter (Yamamura and Snyder 1972).

Choline is then rapidly phosphorylated by choline/ethanolamine kinase (CK) to form phosphocholine (P-choline). Two genes encode CK, CKα and CKβ, and the gene for CKα is alternately spliced to generate two isoforms, CKα1 and CKα2. CKα (1 and 2) and CKβ are found in all murine tissues but a relatively high expression level of CKα is found in liver and testes while CKβ is found primarily in liver and heart. The enzymes are cytosolic, inactive as monomers and can form active homo- or hetero-dimers. CK may also have functions not directly related to PC production: CK protein levels are elevated in response to chemically induced cell stress; P-choline levels are elevated on mitogenic stimulation which appears essential for growth factor signaling and elevated CK protein levels, activity and P-choline are observed in various cancer models (Aoyama et al. 2004). Surprisingly, recent discovery of a mouse mutation in the gene for CKβ points to the importance of the phospholipid biosynthetic pathways for the development and maintenance of healthy muscle tissue (Sher et al. 2006). Rostrocaudal muscular dystrophy, the results of recessive mouse mutation that causes a rapidly progressive muscular dystrophy and a neonatal forelimb bone deformity, is characterized by a complete loss of CKβ protein, reduced CK enzymatic activity in all tissues, and reduced PC levels in fore- and hind-limbs (Sher et al. 2006).

P-choline is converted to CDP-choline by the action of CTP:phosphocholine cytidylyltransferase (CCT) that adds CMP to phosphocholine derived from CTP. This enzyme is rate-limiting for PC formation from this pathway [reviewed in (Vance 2002)]. CCT exists in inactive form in the cytosol and translocates to membrane surfaces where it is active. There are 4 murine isoforms of this enzyme: a ubiquitously expressed CCTα1 and α2 with highest expression levels in lung and testes, and CCTβ1 and β2 which are minor isoforms that are found in brain, lung and gonads (Jackowski et al. 2004). [CCTβ1 cDNA has been found only in human expressed sequence tagged data base (Lykidis et al. 1998)]. A and β isoforms share homology in the catalytic and membrane binding domains, however, the α isoform has a nuclear localization signal and can be detected in the nucleus although the functional significance is unknown [reviewed in (Kent 2005)]. Global knock out of murine CCTα1 is embryonic lethal (Wang et al. 2005) but liver specific knock out of the murine CCTα1 results in decreased apo B and TG secreted into the plasma (Jacobs et al. 2004). It is unknown however if the loss of CCTα1 has a direct effect on lipoprotein production or if the effect on lipoprotein production is secondary to the need to maintain PC homeostasis. CCTβ2 knockout mice, which retain CCTβ3 expression, have gonadal dysfunction such that majority of both homozygous male and female mice are sterile (Jackowski et al. 2004). Both β isoforms are expressed to a limited extent in liver tissue, but the effect of disruption of CCTβ2 expression on lipoprotein metabolism is currently unknown.

CDP-choline:sn 1,2-diacylglycerol cholinephosphotransferase (CPT) is a transmembrane protein found in the nucleus, ER, Golgi, and mitochondrial associated membrane (MAM) and catalyses the final step in PC formation.

I.6.2.2 Methylation pathway

PC can also be derived from methylation of PE by phosphatidylethanolamine methyltransferase (PEMT) in the liver. PEMT utilizes S-adenosylmethionine in a 3 step addition of methyl groups to the ethanolamine moiety of PE. PE can be synthesized from CTP-ethanolamine, in a pathway parallel to the CDP-choline pathway, or via decarboxylation of PS (Figure I.7).

I.6.2.3 CDP-ethanolamine pathway

Ethanolamine is converted to phosphoethanolamine (P-ethanolamine) by either CK (Section I.6.2.1) or specific ethanolamine kinase (EK) 1 or 2. EK1 is uniformly expressed in all tissues examined while EK2 is present largely in kidney, liver, and reproductive tissue. Overexpression of EK1 results in greater utilization of exogenous ethanolamine for PE synthesis yet increases the rate of degradation of PE without effect on PC (Lykidis et al. 2001). Absence of murine EK2 reduces the rate of PE synthesis from exogenous ethanolamine in hepatocytes but has no effect on PE or PC levels. However, pregnant EK2-/- female mice had a 33% reduction in litter size and frequent perinatal death because of the appearance of extensive placental thrombosis at the late stage of pregnancy (Tian et al. 2006).

P-ethanolamine is activated by CTP:phosphoethanolamine cytidylyltransferase (ECT) to form CDP-ethanolamine. PE is then synthesized by a dual-specificity choline/ethanolaminephosphotransferase (EPT) that is located in the ER membrane and is ubiquitously expressed in cells.

I.6.2.4 Decarboxylation of PS to form PE

Serine is an amino acid that is used to synthesize protein. It can be synthesized from phosphoglycerate, an intermediate in glycolysis, and degraded to form pyruvate. Pyruvate can be converted to acetylcoenzyme A, a precursor to fatty acids. Serine can also be converted to glycine and thereby provide a methyl group to tetrahydrofolate. This methyl group can then used to synthesize methionine, which can be converted to S-adenosyl methionine for the methylation of PE to form PC by PEMT. Serine is also a precursor for sphingosine, cysteine and glycerol, the latter of which forms the backbone of phospholipids. Finally, serine is also incorporated into phospholipid as PS.

PS metabolism is reviewed in (Vance and Steenbergen 2005). Decarboxylation of PS to form PE occurs in the mitochondria (Percy et al. 1983; Zborowski et al. 1983). Phosphatidylserine is formed by base-exchange of either choline in PC or ethanolamine in PE for serine by the action of phosphatidylserine synthase 1 (PSS1) or phosphatidylserine synthase 2 (PSS2), respectively. These enzymes are enriched in the MAM, a membrane fraction of the ER closely associated with the mitochondria (Stone and Vance 2000). PS is shuttled to the mitochondria where it is decarboxylated by phosphatidylserine decarboxylase (PSD), generating PE which can then be returned to the MAM or ER. PSS1 cDNA has been isolated and the murine gene characterized (Stone et al. 1998; Sturbois-Balcerzak et al. 2001). The protein is predicted to be multi-membrane spanning and although it lacks a signal sequence at its N terminus, it possesses a C terminal ER retention sequence. When overexpressed in hepatoma cells, PS synthesis is augmented yet the levels of PE and PS are maintained. PSS2 has 30% homology to PSS1 and has an N terminal ER retention signal (Stone and Vance 1999). Overexpression of PSS2 does not change the rate of synthesis of PS and PS-derived PE in contrast to cells expressing similar levels of murine PSS1, supporting the idea that PS synthesis via murine PSS2, but not PSS1, is regulated by end-product inhibition. Moreover, expression of murine PSS2 in these cells did not inhibit PE synthesis via the CDP-ethanolamine pathway, whereas expression of similar levels of PSS1 activity inhibited this pathway by ~50% (Stone and Vance 1999). Both PSS enzymes have broad and overlapping tissue distributions with PSS1 being most abundant in liver, heart, brain, kidney and testis while PSS2 is most highly expressed in testis (Stone and Vance 1999; Sturbois-Balcerzak et al. 2001; Bergo et al. 2002). *Pss2* gene disruption in mouse does not reveal an overt phenotype. However, male knockout mice testes are smaller than those seen in wild type mice and ~10% of the knockout male mice are infertile (Bergo et al. 2002; Steenbergen et al. 2006). Overall, phospholipid composition of different mouse tissues is unaffected when the *Pss2* gene is disrupted; PSS1 activity but not mRNA or protein levels are elevated to compensate for the lack of PSS2 (Bergo et al. 2002; Steenbergen et al. 2006).

Disruption of murine PSD is embryonic lethal (Steenbergen et al. 2005). Elimination of PE production from PS by PSD in mitochondria causes fragmented, misshapen mitochondria, an abnormality that likely contributes to the embryonic lethality and PE produced by the CDP-ethanolamine pathway is unable to compensate for the lack of PSD. PS decarboxylase activity and mRNA levels in tissues of *Psd*+/- mice were approximately one-half of those in wild-type mice. However, the mice appeared normal, exhibited normal vitality, and the phospholipid composition of livers, testes, brains, and of mitochondria isolated from livers, was the same as in wild-type littermates. To compensate, *Psd* heterozygotes maintain PE levels by increasing the activity of the CDP-ethanolamine pathway.

As indicated above, PE produced by decarboxylation of PS may be vital for proper function of the mitochondria and thus viability. Notably, disruption of the gene encoding the murine ECT (*Pcyt2*), the main regulatory enzyme in the CDP-ethanolamine pathway, is also embryonic lethal (Fullerton et al. 2007). *Pcyt2+/-* mice showed a 20-35% decrease in mRNA, protein content, enzyme activity, and decreased rate of PE biosynthesis but maintained phospholipid content without a compensatory increase in the decarboxylation of PS. These results establish the necessity of *Pcyt2* for murine development and demonstrate that a single *Pcyt2* allele in heterozygotes can maintain phospholipid homeostasis.

The contribution of the CDP-ethanolamine pathway relative to decarboxylation of PS as a source of total cellular PE remains to be established. CDP-ethanolamine appears to be the major route of PE synthesis in some mammalian tissue whereas PS decarboxylation is predominant in other tissues and cultured cells [see references cited in (Vance and Vance 2004; Tian et al. 2006)]. It may be that the CDP-ethanolamine pathway is important for maintaining PE and PC for membrane integrity or cellular PC and PE homeostasis but PS is the preferred source of PE for generating PC destined for lipoprotein production (Vance and Vance 1986).

I.6.2.5 Disruption of the murine *Pemt* gene

PEMT is a liver specific ~20-kDa transmembrane protein that catalyzes all three methylation reactions of the conversion of PE to PC (Ridgway and Vance 1987; Ridgway and Vance 1988). Two isoforms of PEMT derived from the same gene have been reported (Cui et al. 1993) although both originate from the same gene (Walkey et al. 1997). PEMT has also been detected in bile canalicular membranes of the liver (Sehayek et al. 2003) and indeed may in part function to maintain bile PC levels during starvation or choline depletion (Vance and Vance 2004).

PEMT deficient (*Pemt-/-*) mice are viable and do not exhibit any obviously abnormal phenotype (Walkey et al. 1997). In the livers of mice homozygous for the deficiency, the amount of PC is comparable to that in wild type mice. The CDP-choline pathway can apparently compensate for the lack of PEMT because the membrane-associated activity of CCT is increased. Because the mice do not show an obvious phenotype when fed laboratory chow, the mice were challenged with a high fat/high cholesterol "Western-style" diet for 3 weeks. Livers from male, but not female, *Pemt–/–* mice accumulated TG and CE compared with wild type mice but the amount of PC was unchanged. Moreover, the plasma TG and apo B were lower in male *Pemt-/-* mice than in wild type mice. The reduction in plasma TG and apo B100 was attributed to a defect in hepatic secretion of VLDL (Noga et al. 2002; Noga and Vance 2003; Noga and Vance 2003). However, the mechanism by which a lack of PEMT inhibits lipoprotein secretion and the basis for an observed sexual dimorphism remain to be elucidated. Thus, it is unknown if this enzyme directly or indirectly affects lipoprotein production.

I.7 Model systems used to study hepatic lipoprotein assembly and secretion

Models used to study apo B lipoprotein assembly include cell free systems, hepatoma cells such as HepG2 and McArdle rat hepatoma 7777 (McArdle RH7777) cells; primary hepatocytes from mice, rats, hamsters, or rabbits, transgenic and gene-targeted animals and as well as whole organs.

The value and history of the use of reconstitution of cell components *in the test* tube is reviewed in (Lingappa and Lingappa 2005). Cellular reconstitution (cell-free systems, CFS) or *in vitro* translation starts with a crude extract made from disrupted cells. Cells are broken open by shearing forces then subject to centrifugation to remove unbroken cells and some large subcellular components. The resulting preparation contains all cytosolic proteins and many membrane-delimited organelles present in the cytoplasm, and is capable of synthesis of proteins *de novo*, when provided specific mRNA, amino acids, energy substrates and other components, and warmed to physiological temperatures. Inclusion of radioactively labeled amino acids makes it possible to distinguish the newly synthesized protein encoded by the added mRNA, from the multitude of other proteins present in the crude extract, by analyzing the extract after completion of protein synthesis by gel electrophoresis. Advantages of this approach include fidelity, focus, and amenability to manipulation. CFS can faithfully reproduce processes that occur within cells. CFS put the spotlight on specific cell processes at greatly slowed rates - likened to slow motion photography - to reveal intermediates and interactions not observed directly, or necessarily looked for, in intact cells. For example, intermediates in the assembly of hepatitis and lentivirus observed using CFS have also been observed in intact cells [see references cited in (Lingappa and Lingappa 2005)]. Furthermore, these systems are amenable to manipulation/perturbation such as accessibility of exogenously added protease to determine topology of a membrane protein (Morimoto et al. 1983).

Rat hepatocytes and McArdle RH7777 cells secrete both apo B48 and apo B100, which differ in their intracellular transit and their assembly into lipoproteins

(Cartwright and Higgins 1995; Rustaeus et al. 1995). The human hepatoma cell line, HepG2 has been extremely useful in studies of translocation and degradation of apo B100; for example see (Dixon et al. 1991; Furukawa et al. 1992; Sakata et al. 1993; Macri and Adeli 1997; Pan et al. 2000). HepG2 cells secrete apo B100 as HDL/LDL sized particles but also exhibit significant differences in lipid metabolism compared to that in normal adult hepatocytes (Gibbons 1994; Gibbons et al. 1994). Similarly, McArdle RH7777 cells secrete higher density apo B containing lipoproteins under basal conditions (Tanabe et al. 1989). These cells lack several enzymes such as PEMT and TGH involved in lipid metabolism (Lehner and Vance 1999; Noga et al. 2002). However, McArdle RH7777 cells are very conducive to cDNA transfection studies (Blackhart et al. 1990). It has been suggested that hepatoma cells lack the second step in the assembly of VLDL, however, provision of exogenous oleate can partially restore this step in McArdle RH7777 cells (Boren et al. 1993). Recent work in Adeli's lab indicates that the HepG2 cells may have an overactive ras-MEK/ERK pathway but that VLDL sized particle secretion can be restored if this pathway is inhibited (Tsai et al. 2007). Thus, although hepatoma cells have limitations, they provide information with respect to the early stages of apo B translocation and lipoprotein assembly.

1.8 Thesis objectives

1.8.1 Role of pause transfer sequences in apo B lipoprotein assembly

High serum cholesterol and apo B are risk factors for atherosclerosis (Sniderman et al. 2000; Sniderman et al. 2001; Sniderman 2002). Apo B is a large hydrophobic protein synthesized by the human liver. Apo B is primarily associated with lipid as VLDL. Similar to other proteins destined for secretion or integration into the ER membrane, apo B is synthesized on ribosomes that associate with the ER. However, apo B is distinctive from other secretory proteins in several ways (Davis and Vance 2002). Apo B must be noncovalently associated with lipid in order to be secreted. Moreover, apo B may possess pause transfer sequences (PTS) that confer a delay, or a transient pause, during translocation into the ER (Chuck et al. 1990; Chuck and Lingappa 1992; Chuck and Lingappa 1992; Chuck and Lingappa 1993). PTS have been characterized in cell-free systems (CFS) (Chuck and Lingappa 1993; Kivlen et al. 1997; Hegde et al. 1998; Rusinol et al. 1998).

Translocational pausing has been observed only in CFS in which the events of translation and translocation are "slowed down" (Hegde and Lingappa 1996). Thus, direct evidence of translocational pausing has not yet been observed in intact cells. The role(s) that translocational pausing may play in apo B-containing lipoprotein assembly is (are) currently unknown. PTS may enable the apo B peptide to interact with resident ER proteins such as chaperones or other factors such as TRAM (Hegde et al. 1998). PTS may also - or instead – provide time for the peptide to associate with lipid, be modified (e. g. by palmitoylation), or interact with other proteins.

In order to understand how apo B translocates into the lumen of the ER, how this process is regulated and the function of PTS, it is first necessary to establish if translocational pausing occurs in intact cells. To achieve this goal intact McArdle RH7777 cells transfected with chimeric proteins containing regions of apo B were examined for evidence of pause transfer.

I.8.2 Localization and source of phospholipid for addition to an apo B lipoprotein

An apo B containing lipoprotein secreted by the liver is a large spherical particle consisting of hydrophobic core of TG and CE surrounded by a 20 Å monolayer of unesterified cholesterol and phospholipid of which phosphatidylcholine (PC) is the major component [reviewed in (Jonas 2002)]. PC is derived from both CDP-choline and from phosphatidylethanolamine (PE) via methylation by phosphatidylethanolamine methyltransferase (PEMT), a function specific to the liver. PE itself is synthesized from either CDP-ethanolamine or decarboxylation of phosphatidylserine (PS) in the mitochondria. PS is synthesized in the ER or the mitochondria associated membrane (MAM) by serine exchange with PC or PE (Vance 2002; Vance and Vance 2004).

The contribution of PC from these pathways to PC secreted as VLDL has been investigated in primary rat hepatocytes (Vance and Vance 1986). These results suggested a preferential utilization of PC obtained from PE derived from decarboxylation of PS (PS is generated from serine exchange either PE or PC). However, the intracellular sites whereby the PC derived from specific phospholipid pools is added to apo B containing lipoproteins have not been determined. It is the objective of the second part of this thesis to determine if there is preferential utilization of PC derived from the specific biosynthetic pathways and to determine the intracellular sites at which the lipid is added using whole rat liver. An advantage of this system is that large quantities of relatively pure intracellular components can be obtained.

Overall, these studies will provide insight as to how apo B containing lipoproteins are assembled and secreted from the liver and potentially provide novel therapeutic targets in order to prevent cardiovascular disease.

II Materials and Methods

II.1 Materials

II.1.1 Animals, cell lines, growth media, antibiotics

Sprague Daley or Long Evan rats were obtained from the animal housing facility on campus. McArdle RH7777 cells were obtained from the American Type Tissue Culture Collection. Dulbecco's modified Eagle's media (DMEM), sera, penicillin G, streptomycin, trypsin-EDTA and neomycin analog G418 were obtained from Life Sciences, Inc.

II.1.2 Antibodies

Mouse anti-prolactin (Prl) polysera was a gift from V. Lingappa (University of California, San Francisco), mouse anti-myc monoclonal, and goat anti-human apo B polysera, rabbit anti-mouse IgG polysera, and mouse anti-goat polysera were obtained from Chemicon. Rabbit anti-calnexin (CXN) and rabbit anti-PDI polysera were obtained from Stressgen. Rabbit anti-rat PEMT was obtained from Denis Vance.

II.1.3 Chemicals and reagents

Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad. Polyvinylidenedifluoride (PVDF) membranes were from Millipore. ECL Western blotting detection system was purchased from Pierce. Protein A-Sepharose CL-4B was obtained from Sigma. Other reagents were purchased from Sigma, Fisher Scientific, or Invitrogen.

II.1.4 Radioactive materials and solutions

[^{35}S]methionine, UDP-N-[^{3}H]acetylglucosamine, [^{3}Hmethyl]choline and Amplify were obtained from NEN Life Scientific.

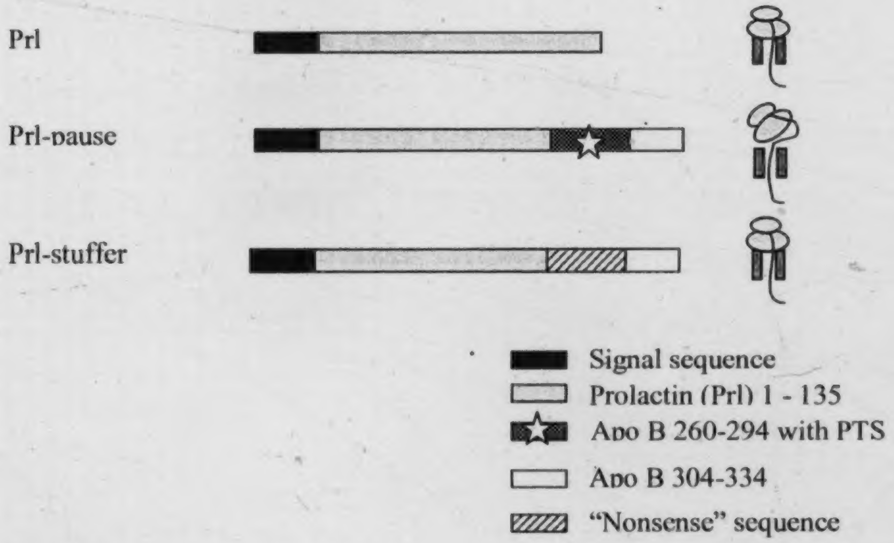
II.2 Methods

II.2.1 Apo B-prolactin plasmids for expression in bacterial cells

Plasmids pSP Prl-pause and pSP Prl-stuffer were provided by V. Lingappa (University of California, San Fransisco) and have been characterized previously (Hedge et al 1998). A diagram outlining the structure of the coding regions of Prl, Prl-pause and Prl-stuffer is shown in Figure II.1.

Briefly, for pSP Prl-pause, the nucleotide sequence for residues 261–290 of mature human apo B was inserted in between the nucleotide sequence for the amino-terminal 165 amino acids (including signal sequence) of bovine prolactin (a typical secretory protein) and the sequences coding for the carboxyl-terminal 304–332 amino acids of mature human apo B. For Prl-stuffer, the coding region for amino acid 261–290 is replaced with the nucleotide sequence encoding a "nonsense" sequence of 30 amino acids. The SP6 promoter drives the expression of Prl, Prl-pause and Prl-stuffer, addition of bacteriophage SP6 RNA polymerase will allow the synthesis of large amounts of mRNA needed for *in vitro* translation (Promega; *in vitro* transcription and RNA isolation are described below). The plasmid pSP Prl, coding for prolactin, a typical secretory protein, was provided by S. Chuck (Harvard Medical School).

Figure II.1 Primary structure of proteins encoded by pSP constructs.
Black box, signal sequence; light grey box, prolactin amino acid sequence 1-135; grey stippled box with star, apo B amino acid sequences 260-294 containing PTS based on (Kivlen et al. 1997), white box, apo B amino acid sequence 304-334; hatched bar, 30 amino acid "nonsense" sequence. For detection in hepatoma cells, cells, a myc tag was appended to the C terminus of Prl-pause and Prl-stuffer (not shown). Models of behavior expected at the ER shown at right; figure eight, ribosome, ribbon, nascent apo B peptide, light grey vertical bars, translocon



II.2.2 *In vitro* transcription and isolation of RNA.

The pSp vectors encoding the sequences described in Section II.2.1 were used to transform *E. coli* and the plasmid DNA isolated by miniprep (Promega) for subsequent RNA preparation and isolation (Promega). Briefly, the plasmids were linearized by restriction enzyme digest, the DNA extracted from the digest by phenol/chloroform, and then combined with SP6 RNA polymerase, RNase inhibitor, ribonucleotides, dithiothreitole, and RNase free water and incubated for 90 min at 40 °C. The DNA was then digested with DNase and the RNA separated from the reaction mixture by phenol/chloroform extraction. The RNA was precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of 95% ethanol and incubation at -70 °C for a minimum of 30 min. The RNA was pelleted by centrifugation at maximum speed (10000 x G) on a microcentrifuge for 10 min at 4 °C, the supernatant removed, the pellet rinsed with 70 % ethanol and then allowed to dry at room temperature. The pellet was then resuspended in RNase free water and the concentration recovered determined by emission spectra at 260 nm versus 280 nm (Sambrook et al. 1989).

II.2.3 *In vitro* translation and protease sensitivity assay.

High levels of mRNA were generated as indicated and translated in the presence of rabbit reticulocyte lysate which is abundant in translation factors and ribosomes, radiolabeled amino acids, and dog pancreas microsomes for 30 min, on ice. Samples were then treated with proteases for 30 min to digest peptides expressed on the outside of the microsomes, leaving lumenal peptides intact. Protease inhibitors (Complete mini, Roche Diagnostics) were added to stop the proteolysis and protect from further digestion following the addition of sample buffer. The samples were incubated at 100 °C for 2-5 min and loaded into the wells of a sodium dodecyl sulfate (SDS) 10% acrylamide gel for electrophoresis (SDS-PAGE). Colourimetric protein standards were loaded alongside the samples to indicate the progress of electrophoresis and subsequent transfer to PVDF membranes.

II.2.4 Gel electrophoresis and transfer to polyvinylpyrolidinefluoride membrane

Acrylamide gels were prepared and run at 100 V for 60-90 min based on the Bio-Rad minigel system. Protein transfer to PVDF membranes was performed at 25 V, 4 °C for 16 hours using the Bio-Rad Western blotting apparatus. The transfer buffer contained 62.5 mmol borate (pH 8.0) after the method of Towbin (Towbin et al. 1979).

II.2.5 Construction of Apo B-prolactin plasmids for expression in mammalian cell lines

Plasmids for expression of Prl, Prl-pause and Prl-stuffer in mammalian cell lines were prepared by sub-cloning the cDNAs described in II.2.1 into pCDNA 3.1 (provided by Zhao Yang, University of Alberta), a mammalian expression vector containing strong CMV promoter and encoding neomycin phosphotransferase, an enzyme that inactivates gentimycin used to generate staple transfectants. A nucleotide sequence including the coding region for a 13 amino acid myc tag was appended to the carboxyl terminus of the Prl-pause and Prl-stuffer in order to facilitate detection by Western blotting.

II.2.6 Generation of transient and stable cell lines

II.2.6.1 Cell culture conditions

DMEM was supplemented with 20% serum (10% fetal bovine serum, 10% horse serum, by volume). McArdle RH7777 cells were maintained at 37 °C in a 5% CO_2, humidified environment in 10 cm tissue culture dishes. Prior to the day of transfection, confluent cells were split into 6 dishes: one dish for positive control using cDNA known to contain the myc tag that had been shown to be expressed in previous work (D. Gilham, personal communication), one control dish for vector alone (to determine that the vector itself is not toxic), one receiving no vector, and the remaining dishes to receive the constructs prepared as described in Section II.2.5.

II.2.6.2 Preparation of transfection mixture

The mammalian expression vector constructs were linearized by restriction digest and combined with N,N-bis[2-hydroxyethyl]-2-amino-ethanesulfonic acid and calcium phosphate and incubated at room temperature for 30 min as described in (Chen and Okayama 1987; Sambrook et al. 1989). The samples were vortexed prior to addition to subconfluent McArdle RH7777 cells.

II.2.6.3 Generation of transient and stable cell transfectants

Cells were incubated for 4 h at 37 °C 5% CO_2 to allow the DNA to adhere to the cells before they were glycerol shocked and then washed with phosphate buffered saline (PBS). The PBS was removed and replaced with media and the cells returned to the incubator. Cells were harvested 24 h later for detection of the expression of the recombinant proteins or subjected to treatment with media enriched with gentamycin over 4-6 weeks to select for stable transfectants. Single cell colonies were gently trypsinized and each colony placed into individual wells of a 6 well culture dish in order to grow to confluence to assess for protein expression. Clones expressing Prl-pause, Prl-stuffer and Prl were then preserved in DMEM, 10% fetal bovine serum, 10% dimethylsulfoxide (v/v) in liquid nitrogen storage.

II.2.7 Characterization of cell lines expressing apo B-prolactin constructs

II.2.7.1 Preparation of cell lysates and media for gel electrophoresis and immunoblot

The media were removed from transfected cells and cells scraped into PBS with a rubber policeman. Cells were placed in separate sets of 15 ml Falcon tubes and subjected to low speed centrifugation (5000 x G) to pellet the cells. The cells were then washed with PBS and re-pelleted, resuspended in 500 μl PBS and transferred to 1.5 ml Eppendorf tubes. The cells were sonicated for 10 seconds at medium speed with an Ultrasonic Processor (model W-385, Heat System-Ultrasonic Inc). Sample buffer was then added to the cell lysate and the samples heated for 2-5 min at 100 °C.

Initially media were not analyzed for transient transfection, largely to conserve antibody for later use once stable cell lines were achieved. The media were transferred to 15 ml tubes and centrifuged at 1000 x G to remove cell debris. The supernatant was either transferred to Amicon filters (10 kDa cut-off) to concentrate the samples and sample buffer added directly, or anti-prl or anti-myc antibody was added and the samples incubated overnight with end over end mixing at 4 °C, followed by the addition of Sepharose A beads to "bring down" the immunocomplexes in order to determine if the recombinant proteins were secreted into the media.

II.2.7.2 Isolation of crude microsomes

Three 10 cm dishes of confluent cells were harvested, resuspended in PBS and either sonicated as indicated in Section II.2.7.1 or homogenized by 6-10 strokes of a Dounce homogenizer. Cell lysates were then centrifuged 10000 x G to pellet cell nuclei and mitochondria. The supernatants were then centrifuged at 99,000 rpm for 20 min in a TLA 100.4 rotor in a benchtop ultracentrifuge at 4 ^{o}C to pellet the microsomes.

II.2.7.3 Carbonate and detergent treatment of microsomal membranes

Microsomes were isolated as described in Section II.2.7.2 and the lumenal contents extracted with carbonate and membranes separated from lumenal contents by ultracentrifugation (Fujiki et al. 1982; Bostrom et al. 1986). The membrane pellet and lumenal contents (supernatant) were analyzed to determine if the PTS appended to Prl causes membrane association. Detergent treatment of the membrane fraction followed by centrifugation established if the chimeric proteins instead form aggregates.

Specifically, the microsome pellet was then resuspended in buffer and divided into 3 portions. One portion was maintained in buffer, one portion subject to 0.1 M or 0.2 M carbonate, and one portion treated with Triton X100 (0.1% final v/v). All reactions were maintained on ice for 30 min, normalized to pH 7 with 6 M hydrochloric acid or acetic acid if necessary, and then re-centrifuged at 99,000 rpm for 20 min in a TLA 100.4 rotor as above. Supernatants and pellets were retained and mixed with sample buffer for analyses by SDS-PAGE and Western blot.

II.2.8 Animal preparation and portal vein injection with [^{3}H]choline

Rats were anesthetized and the liver and portal vein exposed. For some experiments, [^{3}H]choline was injected into the portal vein and the rat kept alive for various times. The livers were harvested and subfractionated to obtain ER and Golgi fractions based on techniques described in Section II.2.9. Lumenal and membrane bound radioactivity and specific phospholipid mass was measured and compared to total organelle protein mass.

II.2.9 Isolation of endoplasmic reticulum and Golgi fractions from rat liver

The livers were rapidly removed and subcellular fractions (two endoplasmic reticulum fractions, designated ERI and ERII, and Golgi vesicles) obtained (Morre et al. 1970) as modified by Hamilton et al (Hamilton et al. 1991) to minimize endosomal contamination of the Golgi membranes (Rusinol et al. 1993; Rusinol et al. 1994). The ERI (rough ER) fraction was isolated from the final discontinuous sucrose gradient at the interface between the sucrose solutions of 1.5 and 2.0 M, whereas ERII (smooth ER) was isolated from the interface between the sucrose solutions of 1.3 and 1.5 M from the same gradient. These fractions were combined to increase total ER yield.

II.2.10 Determination of purity of isolated organelle fractions

II.2.10.1 Enzyme assays

The purity of the isolated membrane fractions was determined on freshly isolated rat liver organelle fractions by assay for NADPH:cytochrome c reductase (ER marker enzyme) and UDP-galactose:N-acetylglucosamine galactosyltransferase (trans-Golgi marker enzyme) as described in (Ernster et al. 1962; Bergeron et al. 1973)

II.2.10.2 Western blot

In addition, purity of the organelle preparation was assessed by Western blot using antibody specific for markers of ER and Golgi (e.g. anti-CXN for ER, anti-TGN38 for Golgi (Tran et al. 2002).

II.2.11 Carbonate extraction of endoplasmic reticulum and Golgi fractions

ERI and ERII from one rat liver was pooled and divided into 8-12 portions while the Golgi was maintained in a single or two portions, depending on yield. The fractions were made up to 3.5 ml with water and 2 M sodium carbonate such that the final concentration of the sodium carbonate was 0.1 M, gently mixed and incubated for 30 min on ice. The lumenal contents were separated from the membranes by centrifugation at 99,000 rpm in a TLA 100.4 rotor at 4°C for 20 min. The pelleted membranes were resuspended in 500 µl PBS and sample buffer to analyze by Western blot the recovery and efficacy of the carbonate extraction, or used for lipid extraction as a control. Lumenal contents were treated with protease inhibitors (Complete mini, Roche Diagnostics) and subjected to immunoprecipitation with anti-apo B polysera (see Section II.2.13).

II.2.12 Determination and optimization of the efficiency of carbonate extraction

Small scale preparations were performed to optimize the efficiency of carbonate extraction as per (Bostrom et al. 1986) or (Fujiki et al. 1982). Lumenal contents were concentrated with trichloroacetate and deoxycholate, dried and both lumenal contents and membranes were resuspended in sample buffer and resolved by SDS-PAGE and then transferred to membranes for Western blot. The presence of marker proteins indicative of each compartment, i.e., PDI for ER lumenal proteins and CXN for ER membrane associated proteins were probed with anti-PDI or anti-CXN polysera, respectively.

II.2.13 Immunoprecipitation of apo B containing lipoproteins

Anti-apo B antisera was added to the supernatant obtained from carbonate extraction, incubated overnight at 4°C with end over end mixing. Protein A Sepharose (Sigma) was added and supernatant mixed for another 2-16 h at 4 °C. The Sepharose beads were pelleted by centrifugation and washed with PBS. Apo B was either extracted by treatment with sample buffer for electrophoresis or lipids extracted as described in the next section.

II.2.14 Lipid extraction

Lipids were extracted as described in (Folch et al. 1957). Essentially 2 parts chloroform to 1 part methanol to 1 part water (sample) were vortexed and the aqueous (upper) phase removed from the hydrophobic (lower) phase after separation by centrifugation.

II.2.15 Thin layer chromatography (TLC)

Samples were dried down with nitrogen gas and resuspended in 50-100 µl chloroform and applied to 20X20 cm silica plates 2 cm from the bottom of the plate. The thin layer chromatograms were resolved in covered glass chambers using a mix of chloroform:methanol:acetic acid:formic acid:water at a ratio of 70:30:12:4:2

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v/v/v/v. Lipid bands were identified by iodine vapour. PC bands corresponding to PC based on a loading standard were scraped from plates for phosphorus analysis.

II.2.16 Phosphorus assay

Standards of 2.5, 5, 10, 20, 30, 40, 60 and 80 nmol phosphate were prepared from stock 0.1 M NaKPO4 and incubated at 180 °C until completely dry. Standards and scraped silica bands were then digested with perchloric acid for 60-90 min at 180 °C. The samples were then diluted to 1 ml volume with water, vortexed to mix and silica pelleted by low speed centrifugation. Portions of the supernatants were then treated with Malachite solution and absorbance promptly measured at 650 nm (Zhou and Arthur 1992).

II.2.17 Determination of radioactivity associated with PC

A portion of the lipid extract or the PC bands scraped from the thin layer chromatogram were mixed with scintillation fluor and radioactivity measured with a Beckman scintillation counter. In some experiments cold phospholipid carrier was added to improve recovery/detection after thin layer chromatography in order to detect the radioactivity incorporated into PC from [^{3}H]choline injection.

II.2.18 Protein concentration determination

Protein concentration was determined using bicinchoninic acid using bovine serum albumin (Pierce) as protein standard and measuring absorbance at 562 nm (Smith et al. 1985).

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III Role of Pause Transfer Sequences in Apo B secretion

III.1 Introduction

III.1.1 Protein transport and apo B containing lipoprotein assembly

Protein transport across the ER membrane into the lumen is an early step in the synthesis of many proteins in eukaryotes (for earlier reviews, see (Hegde and Lingappa 1997; Matlack et al. 1998; Hegde and Lingappa 1999; Johnson and van Waes 1999; Lingappa et al. 2002). Soluble proteins that cross the ER membrane are either destined for secretion or end up localized to the lumen of ER (e.g. protein disulfide isomerase, PDI), Golgi or lysosome. Membrane proteins may be destined to reside in the membranes of the secretory pathway (e.g. calnexin, CXN) or, ultimately, the plasma membrane (e.g. LDL receptor).

Lipoprotein assembly involves apo B synthesis and translocation across the ER membrane, folding, and association with lipids. Apo B may also undergo degradation if conditions do not favour lipoprotein secretion. Evidence has accumulated that the early steps associated with apo B biogenesis are distinct from those of most secretory proteins. For example, unlike most secretory proteins, ER-membrane associated or cytosolic forms of apo B have been detected in rat hepatocytes (Rusinol et al. 1993; Verkade et al. 1993), chicken hepatocytes (Dixon et al. 1992), HepG2 (Davis et al. 1990; Furukawa et al. 1992; Boren et al. 1993), rabbit liver (Wilkinson et al. 1993) and when human apo B is expressed in CHO cells (Thrift et al. 1992). In rat

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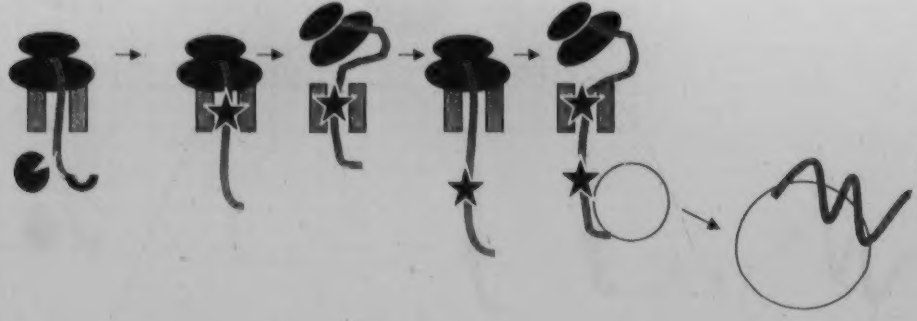
hepatocytes, for example, approximately 50% of the apo B chains are found in a fully translocated form whereas the rest are detected in transmembrane orientation (Davis et al. 1990). It was/is unknown if the cytosolic exposure could be dictated by specific amino acid sequences within apo B.

III.1.2 Pause transfer sequences

The early stages of apo B protein synthesis, targeting and translocation into the ER have been examined more closely in cell free systems (CFS) (Chuck et al. 1990; Pease et al. 1991; Chuck and Lingappa 1992; Chuck and Lingappa 1992; Chuck and Lingappa 1993; Pease et al. 1995; Rusinol et al. 1998). Some of these studies further reinforce that apo B may not behave like a typical secretory protein (Chuck et al. 1990; Chuck and Lingappa 1992; Chuck and Lingappa 1992; Chuck and Lingappa 1993; Kivlen et al. 1997; Rusinol et al. 1998). That is, whereas a typical secretory protein like Prl is cotranslationally translocated directly into the ER lumen and the ribosome maintains a tight seal, apo B may transiently behave like a membrane protein even though it lacks hydrophobic α helical domains typical of transmembrane proteins (Yang et al. 1989). Instead, apo B has been reported to contain pause transfer sequences (PTS) that direct the transient stopping and subsequent restarting of its translocation, a phenomenon referred to as translocational pausing (Chuck et al. 1990; Chuck and Lingappa 1992; Chuck and Lingappa 1992). During translocational pausing of apo B, the ribosome-membrane junction and ER translocation channel are altered in such a way as to expose the nascent polypeptide to the cytosol (Hegde and Lingappa 1996). Thus, the ribosome may tilt to allow the exposure of the peptide to the cytosol or the nascent peptide protrudes through a gap between the ribosome and the translocon while translation proceeds. These two models are depicted in Figure III.1 A and B.

Figure III.1 Effect of PTS on translocation of apo B.
Models depict how apo B might enter the ER: For apo B the first steps are similar to a typical secretory protein with mRNA encountering the ribosome and synthesis of the signal sequence (dark grey). The ribosome peptide complex is then targeted to the ER and the peptide begins to enter the lumen and the signal sequence is cleaved (Pacman represents signal peptidase). As translation proceeds, a PTS is encountered (star) and translocation is halted while translation continues, the ribosome tilts exposing the peptide to the cytosol (A.) or the peptide is exposed to the cytosol via a gap in the junction between the ribosome and the translocon (B.). Translocation of the peptide resumes and translation/peptide entry into the ER lumen continues. The cycle of translocational pause and cytosolioc exposure is repeated on encounter of the next PTS (second star) and so on until the peptide is fully synthesized and a lipoprotein is produced.

A. ribosome tilting



B. gap between ribosome and channel

B. gap between ribosome and channel

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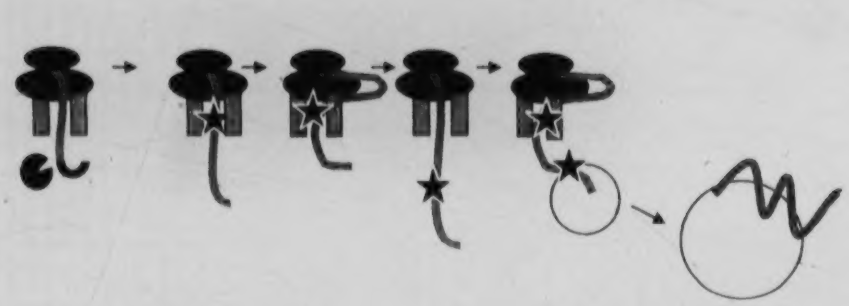
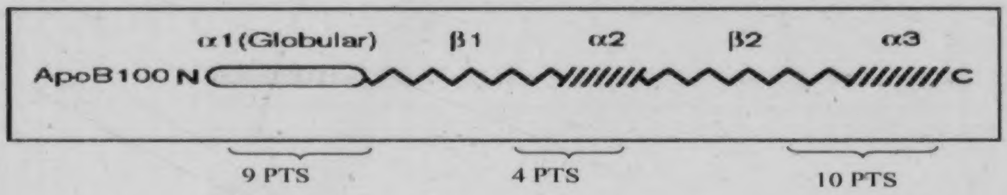


Mutagenesis studies indicate the sequence requirements for PTS may consist of the consensus sequence LKK - T- - - - N - - - A (dashed lines refer to flexibility in the amino acid sequence requirements), although this information was based on analysis of a single 40 amino acid segment containing a putative PTS (Chuck and Lingappa 1992; Chuck and Lingappa 1993). Furthermore, PTS are unevenly distributed throughout apo B, specifically, 23 of the 50 PTS predicted demonstrate pause transfer activity in cell free assays (Kivlen et al. 1997) and may reflect the importance of flanking sequences in order to function. From this analysis, the "functional PTS" appear to congregate in the amphipathic α helical regions of apo B predicted by Segrest and colleague (Figure III.2) although the significance of this distribution has yet to be appreciated (Kivlen et al. 1997). Functional PTS have also been found in ER-resident protein BiP (Chuck and Lingappa 1993; Hegde et al. 1998), prion proteins (Nakahara et al. 1994), the trabecular meshwork glucocorticoid response (TIGR) protein (also called myocilin), another secretory protein (Zimmerman et al. 1999), and in transmembrane protein γ-aminobutyric acid transporter GAT-1 which produces transient intermediates and has the PTS consensus sequence (Clark 1997).

Figure III.2 Distribution of PTS throughout apo B

From (Kivlen et al. 1997) superimposed on the pentapartite structure proposed by (Segrest et al. 1994). Image from (Shelness and Sellers 2001).



Fusion protein and proteoliposome reconstitution studies show that a PTS-containing protein, but not a PTS-deficient protein, interact with an 11 kDa unidentified glycosylated transmembrane protein (Hegde and Lingappa 1996) and translocating peptide associated membrane protein, TRAM (Hegde et al. 1998). Results from the latter experiment suggested that TRAM may regulate the exposure of the peptide to the cytosol and therefore serve as part of the control of apo B secretion (Hegde et al. 1998).

The role of PTS in apo B containing lipoprotein assembly and secretion is unknown. A critical issue is whether protein synthesis and subsequent events observed in CFS occur in a manner that faithfully reflects what occurs within living cells (Lingappa and Lingappa 2005). In the investigations cited above, pausing has been observed only in CFS. Pausing has yet to be observed directly *in vivo*. It is speculated that PTS may enable to apo B peptide to interact with resident ER proteins or allow the peptide time to interact with lipid or be modified. PTS may also, or instead, permit intra-molecular interactions to occur (e.g. between α helical bundles) that may then facilitate lipid binding.

instead, permit intra-molecular interactions to occur (e.g. between α helical bundles)
that may then facilitate lipid binding.

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III.1.3 Hypothesis

In order to determine how apo B translocates into the lumen of the ER, how this process is regulated and the function of PTS, it is first necessary to establish if translocational pausing can directly confer a phenotype such as delayed secretion or cytosolic exposure when expressed in cells. To achieve this goal, hepatoma cells were transfected with a construct encoding a fusion protein consisting of Prl, a typical secretory protein, and a region of apo B shown previously to have a single PTS (Prl-pause). These cells were compared to cells expressing a fusion protein of Prl and a "nonsense" sequence (Prl-stuffer) or Prl alone. It was hypothesized that the expression of Prl-pause would, by virtue of the PTS, demonstrate impaired secretion relative to Prl or Prl-stuffer. Results indicated that Prl-pause demonstrated pause transfer activity in CFS while the other two constructs did not. All 3 constructs could be detected intracellularly but only Prl could be detected in the media. Prl-pause and presumably Prl-stuffer may instead form aggregates in the microsomal lumen. Therefore it was concluded that the system of study might not be suitable to address the role of PTS in apo B containing lipoprotein assembly and secretion.

III.2 Results

III.2.1 Prl-pause is partially exposed to the cytosol *in vitro*

Prl-pause, Prl-stuffer and Prl have been previously characterized (Hegde et al. 1998); the structure of these constructs is depicted in Figure II.1 of Chapter II. Prl-pause consists of the apo B amino acid sequences 261-290 inserted between first N terminal 165 amino acid sequences of Prl (includes signal sequence) and apo B

terminal 165 amino acid sequences of Prl (includes signal sequence) and apo B

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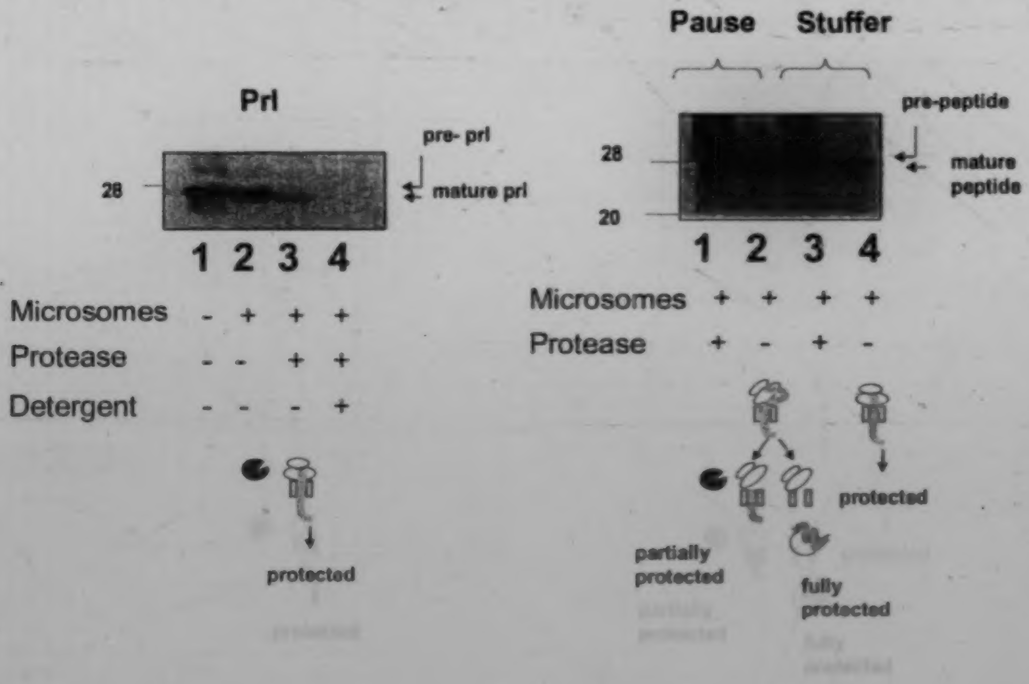
sequence 304 -334. Prl-stuffer the amino acid 261-290 sequence is replaced with a "nonsense" sequence of 30 amino acids. Previous experiments indicated that Prl-pause has pause transfer activity in CFS while Prl-stuffer has not (Hegde and Lingappa 1996). These results were confirmed by standard *in vitro* translation and protease accessibility assay using dog pancreas microsomes (Figure III.3).

Figure III.3 Prl-pause demonstrates pause transfer activity *in vitro* while Prl and Prl-stuffer do not.

RNA was incubated with [^{35}S]methionine and rabbit reticulocyte lysate in the presence (+) or absence (-) of microsomes, protease or detergent. The products were resolved by SDS-PAGE and autoradiographed. Upward pointing arrow in lane 7 indicates partially protected fragment observed in the presence of lysate, microsomes and protease for Prl-pause. [20 and 28 are standard molecular mass markers (kDa)]. Models depicted below the autoradiograms indicate the effects of exogenous protease (Pacman). The experiment is a representative of one of two experiments.



High levels of mRNA were generated and translated in the presence of translation factors, radiolabeled amino acids and microsomes. Samples were then treated with protease. It was observed that Prl-pause but not Prl-stuffer or Prl, by virtue of pausing, resulted in the partial cytosolic exposure of the fusion peptide, the exposed portion of which was susceptible to the exogenously added proteases whereas the N terminal fragments remained protected within the ER lumen and translocon channel (Figure III.3 upward pointing arrow). As expected, Prl and Prl-stuffer, both lacking PTS, were fully translocated and therefore protected from protease in this assay. As a control, the microsomal membranes were dissolved with detergent, rendering Prl completely susceptible to protease. This result suggests that the microsomes were intact and the protease completely active.

III.2.2 Generation of stably transfected McArdle RH7777 cells expressing Prl-pause, Prl-stuffer, and Prl.

III.2.2.1 Prl-pause, Prl-stuffer, and Prl can be detected in cell lysates

Prl-pause and Prl-stuffer were inserted into a mammalian expression vector that includes the nucleotide sequences such that a myc tag is appended to the C terminus of the protein to be expressed. A mammalian vector expressing Prl was provided by S. Chuck (Harvard Medical School). McArdle RH7777 cells were transfected with the plasmids encoding Prl-pause, Prl-stuffer and Prl as described in Chapter II. Transient cellular expression of Prl-pause and Prl-stuffer in rat hepatoma cells was detected by anti-myc antibody as well as anti-Prl antisera. Prl (lacking a myc tag) was detected with the anti-Prl antisera. As shown in Figure III.4, proteins of the predicted molecular mass (28 kDa for Prl-pause and Prl-stuffer, 25 kDa for Prl)

the predicted molecular mass (28 kDa for Prl-pause and Prl-stuffer, 25 kDa for Prl)

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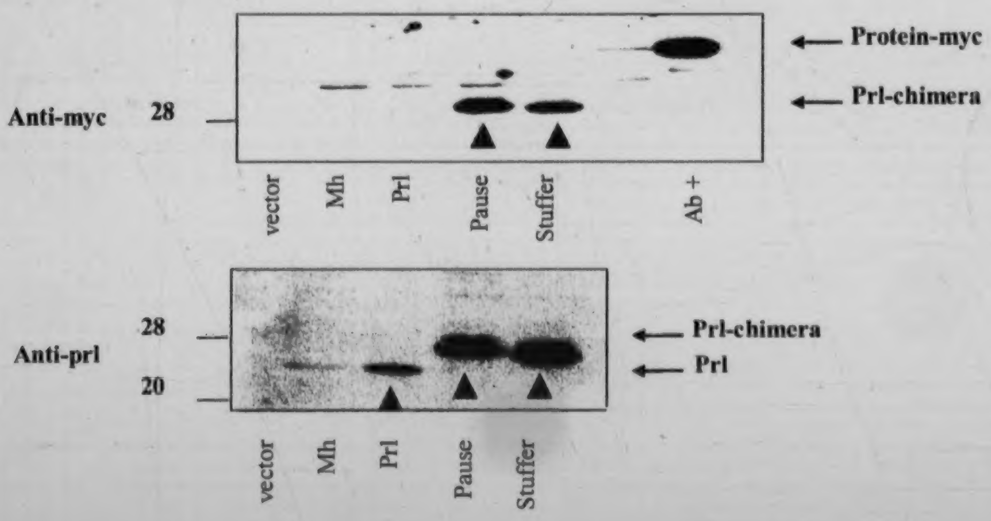
were detected. Prl can be detected in the media of transfected cells while Prl-pause and Prl-stuffer can not.

Figure III.4 Prl-pause, Prl-stuffer and Prl can be detected in transiently transfected McArdle RH7777 cells.

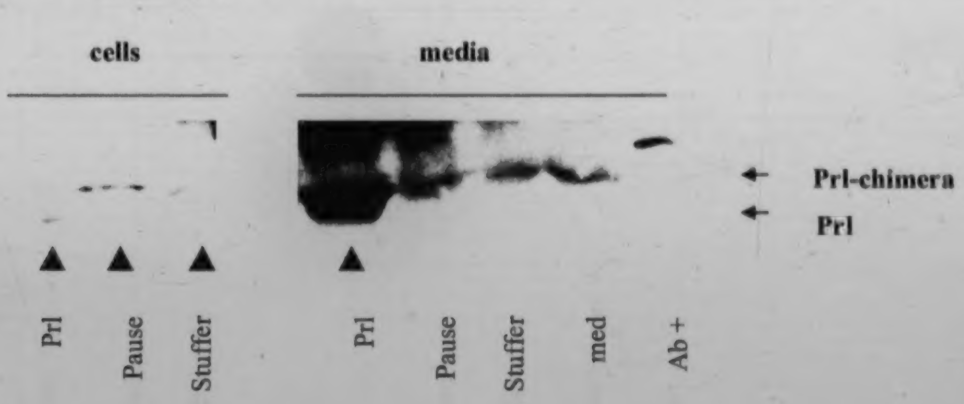
Cell lysate from transfected cells were treated with sample buffer and loaded onto SDS-PAGE followed by transfer to PVDF membrane as described in Chapter II. The PVDF membrane was then probed with anti-myc antibody, stripped then reprobed with anti-Prl anti-serum. **Vector**, pCDNA.1 without insert used to transfect cells; **Mh**, untransfected McArdle RH7777 cells; **Prl**, pCDNA.1 with Prl insert used to transfect cells; **Pause**, pCDNA.1 with Prl-pause insert used to transfect cells; **Stuffer**, pCDNA.1 with Prl-stuffer insert used to transfect cells; **Ab+**, control for myc antibody (**protein-myc**, myc tagged protein, provided by D. Gilham). The results shown are from a single experiment.



Stable cell lines were then generated and cryo-preserved for future use as described in Chapter II, Section II.6.3. Three representative clones were revived, tested for expression and used to determine if the proteins were secreted. Various attempts to immunoprecipitate Prl-pause and Prl-stuffer proteins in the media have failed (Figure III.5 and data not shown). However, Prl protein could be immunoprecipitatated from the media of Prl expressing cells (Figure III.5).

Figure III.5 Prl, but not Prl-pause or Prl-stuffer, is detected in the media of stably transfected cells.

Cells expressing Prl, Prl-pause or Prl-stuffer were plated on 10 cm dishes, allowed to attach to the plate overnight, and the media removed and 5 ml fresh media added and the cells incubated for another 24 h such that the cells were confluent by the end of the experiment. Cells and media were harvested. Cells were resuspended in PBS and sonicated for 10 seconds. Media were centrifuged to remove cells and debris, and incubated with anti-Prl antisera. One tenth of the cell lysate and all the media immunoprecipitates were then resolved by SDS-PAGE, transferred to PVFD membrane, and probed with anti-Prl antisera. Light grey upward facing arrows indicate Prl, upward facing dark grey arrows indicate Prl-pause and Prl-stuffer. Results shown are from one of several experiments. **med**, unconditioned media; **Ab +**, cell lysate from a previous experiment shown to be detected by the anti-Prl antisera.(antibody control)



III.2.2.2 Prl-pause may form aggregates in the lumen of the ER

The microsomal fraction from cells expressing the Prl-pause, Prl-stuffer and Prl was isolated to determine if the proteins were lumenal, membrane associated, or had formed aggregates. Microsomes were isolated from the stable cell lines and treated with carbonate to separate lumenal contents from membrane associated proteins by the method of (Fujiki et al. 1982), described in Chapter II. Alternately, the microsomes were treated with detergent to solubilize the membrane proteins. Protein complexes or aggregates refractory to solubilization were then pelleted by ultracentrifugation. Results suggested that Prl, Prl-pause and Prl-stuffer were present in both the membrane and lumenal fractions after carbonate extraction (Figure III.6 A., lanes 1 and 2 of Prl and Pause panels; also data not shown). CXN, an ER resident membrane protein, was primarily distributed in the membrane pellet while most of the PDI, an ER resident lumenal protein, was associated with the supernatant rather than the pellet after carbonate extraction, indicating that the carbonate treatment was successful in separating membrane associated from lumenal proteins (Figure III.6 B. lanes 1 and 2 of CXN and PDI panels). The Triton X100 was also capable of solubilizing the membrane associated CXN such that it was found in the supernatant after treatment with this detergent Figure III.6 B., lanes 3 and 4 of CXN and PDI panels). Prl, which had been detected in the media of cells expressing this protein, appeared to be associated with both the lumen and the membrane fractions after carbonate treatment (Figure III.6 A. Prl panel lanes 1 and 2). However, detergent treatment of the microsomes released most of the Prl into the supernatant (Figure III.6 Prl panel lanes 3 and 4) while Prl-pause was found in both the pellet and the

Prl panel lanes 3 and 4) while Prl-pause was found in both the pellet and the

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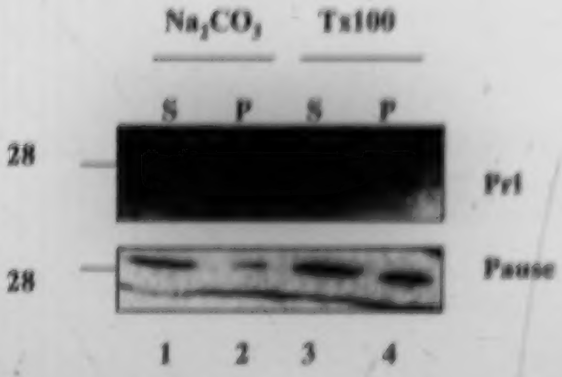
supernatant Figure III.6 A. Prl-pause panel lane 3 and 4), suggesting that the latter had formed complexes or aggregates in the ER. This experiment needs to be repeated to verify these results and Prl-stuffer must also be analysed. Prl-stuffer is expected to resemble Prl-pause under these conditions although this experiment needs to be done.

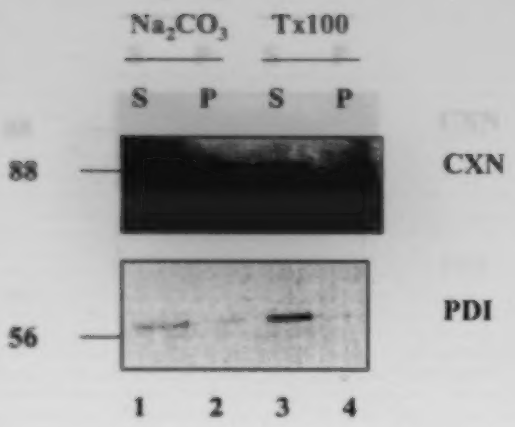
Figure III.6 Prl-pause may form aggregates while Prl does not.

Microsomes from stably transfected cells expressing Prl-pause or Prl were isolated and treated with carbonate (Na_2CO_3) or detergent (Tx100). The reactions were ultracentrifuged at 99,000 rpm in a TLA 100.4 rotor for 20 min and the pellet resuspended. Both the supernatant and pellet (S and P, respectively) were treated with sample buffer and loaded onto SDS-PAGE followed by transfer to PVDF membranes. **A.** The membranes were probed with anti-prl antisera to detect Prl-pause (Pause panel) or Prl (Prl panel) **B.** The membranes were probed with anti-CXN (CXN panel) or anti-PDI (PDI panel).

A.



B.



III.3 Discussion

III.3.1 Summary

Rat hepatoma cell lines stably expressing Prl-pause, Prl-stuffer and Prl were generated in order to determine if proteins containing PTS expressed in living cells demonstrate a phenotype such as delayed secretion relative to control and to determine if the behavior of PTS containing protein in cells resembles the findings from CFS studies. It was found that while Prl-pause demonstrated pause transfer activity *in vitro*, when expressed in cells, the protein was not secreted and may instead formed aggregates, suggesting that the protein was malformed, although this conclusion requires further verification. Prl was protected from protease digestion *in vitro* as expected, and was secreted by the transfected cells.

III.3.2 Pause to consider

In vitro studies have been used to determine events that occur at the early stages of apo B protein synthesis, targeting and translocation into the ER (Chuck et al. 1990; Pease et al. 1991; Chuck and Lingappa 1992; Chuck and Lingappa 1992;

Chuck and Lingappa 1993; Pease et al. 1995; Chen et al. 1998; Rusinol et al. 1998). Some of these studies indicate that apo B may not behave like a typical secretory protein (Chuck et al. 1990; Chuck and Lingappa 1992; Chuck and Lingappa 1992; Chuck and Lingappa 1993; Kivlen et al. 1997; Rusinol et al. 1998). Other investigations counter that apo B is exposed to the cytosol either *in vitro* or when expressed in cells (Pease et al. 1991; Shelness et al. 1994). Instead, apo B may be entirely inserted into the inner leaflet of the ER membrane without the formation of paused intermediates (Pease et al. 1991). Alternately, apo B may be completely translocated into the ER lumen, with little membrane association at all (Shelness et al. 1994). When 300 amino acid length overlapping regions of apo B48, apo B28 and apo B53 were appended to either the native N terminal signal sequence of apo B or a heterologous signal sequence were expressed in COS-1 cells, none of the peptides demonstrated transmembrane topology and only the segments from the N terminus of apo B were secreted by these cells. This result led the investigators to conclude that translocation of apo B was not impaired (Shelness et al. 1994). However, COS-1 cells do not produce apo B normally therefore may lack components present in liver and intestinal cells such as MTP that might arrest translocation of apo B into the lumen of the ER. Indeed, co-expression of MTP with human apo B in COS-1 improves the secretion of the apo B constructs although whether or not the intracellular apo B peptide is exposed to the cytosol was not investigated in this study (Leiper et al. 1994).

Ribosomal pausing has also been put forth as an explanation for the unusual intracellular topology of the apo B peptide (Pease et al. 1995; Leiper et al. 1996).

This process would involve transient translation arrest rather than translocational pausing (Pease et al. 1995; Leiper et al. 1996). In this instance, the ribosome would encounter a signal to stall, and translation would be uncoupled from translocation such that the apo B peptide would be forced to become cytosolically exposed by virtue of the attached ribosome. Translational pausing sequences create apo B intermediates of variable size in the presence or absence of microsomes because of variable re-initiation of translation. These sequences occur within the first 9 % of apo B and bear similarity to the PTS consensus sequence (Pease et al. 1995), or in 2 regions of the C terminus (Leiper et al. 1996), *in vitro*. However, ribosomal pausing did not lead to cytosolic exposure when fusion constructs were expressed in COS-1 cells, although it is unknown if these proteins can be secreted (Pease et al. 1995). Furthermore, partial disruption of microsomes obtained from these cells with low concentrations of saponin generated fragments on treatment with protease, suggesting that the intermediates observed in Lingappa's laboratory may be a consequence of proteolysis and leaky microsomes (Leiper et al. 1996). In contrast, Chuck and Lingappa (1992) have observed PTS only when microsomes were included in the CFS, when translation was uncoupled from translocation by the addition of puromycin to the CFS (terminating translation and releasing the newly synthesized peptide), or by the addition of microsomes to the CFS after the apo B peptide had already been translated (Chuck and Lingappa 1992). Moreover, to eliminate the concern of generating partial protein fragments from indiscriminant proteolysis or leaky microsomes, myc-tagging apo B15 (representing the N terminal 15% of apo B) at various locations or inserting a site specific protease recognition sequence also revealed cytosolic exposure of apo B15, presumably due to the presence of PTS (Hegde and Lingappa 1996).

The fusion proteins used in this thesis had also been investigated by (Hegde and Lingappa 1996). These investigators showed that Prl-pause was partially susceptible to protease while the other constructs were not, which was confirmed here. Prl-pause was also found to bind an 11 kDa protein while Prl-stuffer did not (Hegde and Lingappa 1996). Later, it was shown that TRAM was required for proper assembly of a translocationally paused nascent chain (Hegde et al. 1998). In the absence of TRAM, both Prl-pause and Prl-stuffer are inappropriately exposed to the cytosol while in the presence of TRAM, only select domains of Prl-pause are exposed. However, Lingappa and colleagues had never expressed these constructs in cells to determine if these proteins could be secreted or associate with membranes within cells, an approach usually used to confirm results observed in CFS (see Section I.7 of the Introduction). It was the object of this thesis to determine if Prl-pause displayed a phenotype such as membrane association or impaired secretion when expressed in cells. Cells expressing Prl could secrete Prl protein but it did not appear that Prl-pause and Prl-stuffer were secreted although further work is required to quantitate or more precisely detect levels of the proteins in cells and media (e.g. radioactive labeling). Oddly, although the Prl protein was detected in the media, Prl was also associated with the microsomal membrane after carbonate treatment and could be released by treatment with detergent, perhaps suggesting less than typical behaviour of a "typical" secretory protein. It must be noted that only single representatives of the stably transfected cell lines were investigated in this thesis. It might be possible that the results observed were due to clonal variation, high levels of expression, or a position effect might be contributing to the phenotypes observed. Therefore, other cell lines should be revived and tested to confirm these results.

It is yet unclear why there is a discrepancy between the results from different laboratories. However, the apo B substrates used in several of these studies have included the N terminal 15 % of apo B or portions thereof which, based on Kivlen et al (Kivlen et al. 1997), may include up to 4 PTS as well as β domains based on predictions made by Segrest's laboratory (Segrest et al. 1994) and reviewed in (Smolenaars et al. 2006). Thus it is not clear if PTS are directly involved or, as discussed in the next section, if β sheet domains predominantly dictate if apo B will be exposed to the cytosol.

III.3.3 PTS versus β sheet domains in translocation of apo B

Transient exposure of apo B to the cytosol may instead be mediated by β sheets present in the apo B peptide (Liang et al. 1998; Yamaguchi et al. 2003; Yamaguchi et al. 2006). While the experiments reported in this thesis were underway, similar experiments were performed in Ginsberg's laboratory (Liang et al. 1998; Yamaguchi et al. 2003; Yamaguchi et al. 2006). Albumin was fused with regions of itself, with apo B regions containing two PTS, with single β sheet regions or with both a PTS and a β sheet. These investigators found that β sheets were exposed to the cytosol, associated with Sec61, and were ubiquinated. Moreover, the proteins were secreted much less efficiently than the control albumin-albumin fusion protein. Overall, all the constructs, including albumin-albumin, were less efficiently secreted than intact albumin. In the presence of PTS however, the proteins were fully

translocated into the ER lumen and were nearly as efficiently secreted as the albumin-

translocated into the ER lumen and were nearly as efficiently secreted as the albumin-albumin control. Unexpectedly, treatment with oleate augmented the secretion efficiency of PTS containing proteins but not β sheet containing proteins. The explanation for this observation is unknown. These investigators suggested that selection of a region of apo B containing two PTS (apo B12-17) used in their study might somehow negate effects seen if only one PTS were used. Thus it may be possible that addition of oleate to the transfected cells generated in this thesis investigation might result in the detection of Prl-pause in the media while Prl-stuffer would remain retained within the cells. Prl secretion would be expected to be unaffected by the treatment with oleate.

Notably, when phosphatidylmonomethylethanolamine (PMME) enriched microsomes were utilized in the reconstitution of apo B15 translocation *in vitro*, it was found that compared to untreated microsomes, restart of translocation of apo B15 was prevented, presumably due to the presence of PTS (Rusinol et al. 1998). Again, it should be noted that apo B15 contains other domains that might be responsible for this response (see above). In this thesis investigation, the topology of the fusion proteins relative to the ER membrane from the transfected cells was not determined. Presumably, based on the results of Yamaguchi et al (Yamaguchi et al. 2006), it might be expected that Prl-pause would not be exposed to the cytosol, associated with the Sec61 translocon, or ubiquinated, unless a β sheet is also appended. A single experiment was attempted in this thesis with monomethylethanolamine (MME) treatment of the transfected cells. However, the agent proved to be toxic and the experiment was not repeated. One could postulate that the translocation impairment observed with PTS *in vitro* might only become apparent if the ER membrane was enriched in PMME. Prl-pause would then become exposed to the cytosol while the other constructs would not. It is uncertain how the constructs designed by Yamaguchi et al. (2006) would respond to MME treatment. The inclusion of a β sheet seemed to result in cytosolic exposure regardless of condition. It is possible that the PTS containing constructs in their study might become exposed to the cytosol on MME treatment of these cells. Finally, these investigators did not determine if TRAM is associated with any of the fusion proteins they generated. It is possible that PTS containing constructs might cross-link with TRAM or the 11 kDa protein described earlier, although what effect these interactions would have is unknown. The role of TRAM in apo B lipoprotein assembly and secretion merits further investigation. Approaches to determine if TRAM interacts with apo B might be by cross-linking or co-immunoprecipitation experiments, or knock down or overexpression of TRAM in cells expressing apo B. It might be expected that in the absence of TRAM, based on the *in vitro* studies (Hegde et al. 1998), more regions of apo B might be exposed to the cytosol, since it would be inefficiently incorporated into the ER lumen, in contrast, overexpression of TRAM would increase lumenal association of apo B.

Protease protection studies to determine the topology of integral membrane proteins have shown that data on individual fusion proteins may be misleading [(Skach and Lingappa 1993), also reviewed in (Ott and Lingappa 2002)]. The same may apply to apo B with respect to PTS. Thus cooperative interactions of other domains such as β sheets may be required to manifest cytosolic exposure of apo B.

Another caveat to this approach is the artificial nature of these hybrid proteins and the well known capacity of the ER and post ER intermediate compartment to participate in retention and recycling of misfolded and unassembled proteins [reviewed in (Pelham 1989; Trombetta and Parodi 2003)]. Such an approach might preclude drawing conclusions about apo B domains that may promote or disrupt intracellular transport. This situation may be the case at least for Prl-pause, since it could be detected in a pellet after treatment with detergent, suggesting the protein formed complexes or aggregated in the ER lumen and that therefore, the protein might be malformed. Therefore, this system might not be suitable for the investigation of the role, if any, of PTS in lipoprotein assembly.

IV Source and sites of phospholipid addition to apo B containing lipoproteins in liver

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IV.1 Introduction

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IV.1.1 Apo B lipidation

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Apo B consists of two forms, apo B100, which is produced by the human liver,

Apo B consists of two forms, apo B100, which is produced by the human liver, and apo B48, which is produced in the human intestine by site-selective mRNA editing such that a stop codon is introduced to result in a peptide that is co-linear with the first amino terminal 2152 amino acids of apo B100 (Innerarity et al. 1996; Rustaeus et al. 1999; Blanc and Davidson 2003). In mice and rats, both apo B48 and apo B100 are synthesized and secreted by the liver. Based on electron microscopy studies of liver and brefeldin A treatment of hepatoma cells, lipoprotein synthesis involves at least two steps: co-translational lipidation results in the formation of a primordial lipid particle which then might fuse with or acquire lipid from a lumenal lipid droplet (Alexander et al. 1976; Rustaeus et al. 1999). This process may occur in the lumen of the ER (Alexander et al. 1976; Borchardt and Davis 1987; Rusinol et al. 1993; Kulinski et al. 2002; Yamaguchi et al. 2003) or during transport and/or within the Golgi (Janero and Lane 1983; Bamberger and Lane 1988; Bamberger and Lane 1990; Olofsson et al. 2000; Swift et al. 2001; Tran et al. 2002; Valyi-Nagy et al. 2002). Many of these studies largely focused on bulk lipid addition without distinction to the separate lipid classes or without considering the possibility of lipid modification or remodeling during the assembly and secretion of the lipoprotein.

IV.1.2 Apo B and phosphatidylcholine

Phosphatidylcholine (PC) is the major phospholipid in lipoproteins secreted by the liver (Jonas 2002). The synthesis of this lipid is reviewed in Chapter I, Section I.6 and depicted in Figure I.6. PC is derived from 2 different pathways in the liver: the Kennedy pathway via CDP-choline; and from phosphatidylethanolamine (PE) via methylation by phosphatidylethanolamine methyltransferase (PEMT), a function specific to the liver. PE itself is synthesized from either CDP-ethanolamine in the ER or decarboxylation of phosphatidylserine (PS) in the mitochondria (see Figure I.6, Chapter I). PS is synthesized in the ER or the mitochondria associated membranes (MAM) by exchange of serine with the choline moiety of PC or the ethanolamine moiety of PE, catalysed by phosphatidylserine synthase 1 (PSS1) or phosphatidylserine synthase 2 (PSS2), respectively (Vance 2002; Vance and Vance 2004). Few studies have examined how and where phospholipids are assembled into an apo B lipoprotein in the liver. Pulse chase analysis of chicken hepatocyte lipoprotein phospholipids using [^{3}H]palmitate resulted in a biphasic addition of phospholipid, suggestive of early addition in the ER and then later in the Golgi (Janero and Lane 1983). Higgins and Hutson isolated ER and Golgi fractions from [^{3}H]palmitate-labeled rat liver and separated the membrane from cisternal contents for analysis (Higgins and Hutson 1984). These investigations, similar to results with chicken hepatocytes, indicated that the TG component of the VLDL is sequestered into the cisternae of the ER with a small amount of phospholipid while the bulk of the phospholipid is packaged with the TG in the Golgi cisternae. However, these studies could not unequivocally morphologically pinpoint the sites of specific phospholipid addition nor the origin or type of phospholipid. In contrast, other studies have indicated that the lipoproteins are fully assembled in the ER (Rusinol et al. 1993; Yamaguchi et al. 2003).

Phospholipid may be synthesized in the ER and transferred as a component of membrane vesicles to the Golgi membrane for packaging with TG as a lipoprotein or, alternatively, phospholipids destined for secretion may be synthesized in the Golgi membranes and subsequently transferred into cisternal space and packaged with the TG transported from the ER. A later study by Higgins and Fieldsend (Higgins and Fieldsend 1987) indicated that [^{14}C]choline- and [^{3}H]methionine-labeled precursors were incorporated into rat liver Golgi membranes *in vivo* at rates inconsistent with transfer of phospholipid from the ER. Furthermore, there was preference for the methylation pathway for lipoprotein destined PC, although whether the PE was derived from ethanolamine or decarboxylation of PS was not established (Higgins and Fieldsend 1987). These investigators also established that the enzymes involved in the final steps of the CDP-choline and methylation pathway for the synthesis of PC were found at relatively high levels in the Golgi fractions. CTP:phosphocholine cytidylyltransferase, CCT, which catalyzes the rate-limiting step in the CDP-choline pathway, was measured under the same conditions and also found to be present in isolated Golgi at levels comparable with that of the ER (Higgins and Fieldsend 1987). A separate study also showed that the Golgi has the capacity to make PC via the CDP-choline and the methylation pathways, PS by base-exchange, and PE by the CDP-ethanolamine pathway but not by decarboxylation of PS (Vance and Vance 1988). Finally, the contribution of PC from CDP-choline, ethanolamine and serine to

PC secreted as VLDL has been investigated in primary rat hepatocytes (Vance and Vance 1986; Vance 1988). The results of these studies indicated a clear preference for secretion of PC derived from choline or serine, and PE made from serine (Vance and Vance 1986). However, the intracellular site whereby the PC derived from specific phospholipid pools is added to the lipoprotein had not been determined.

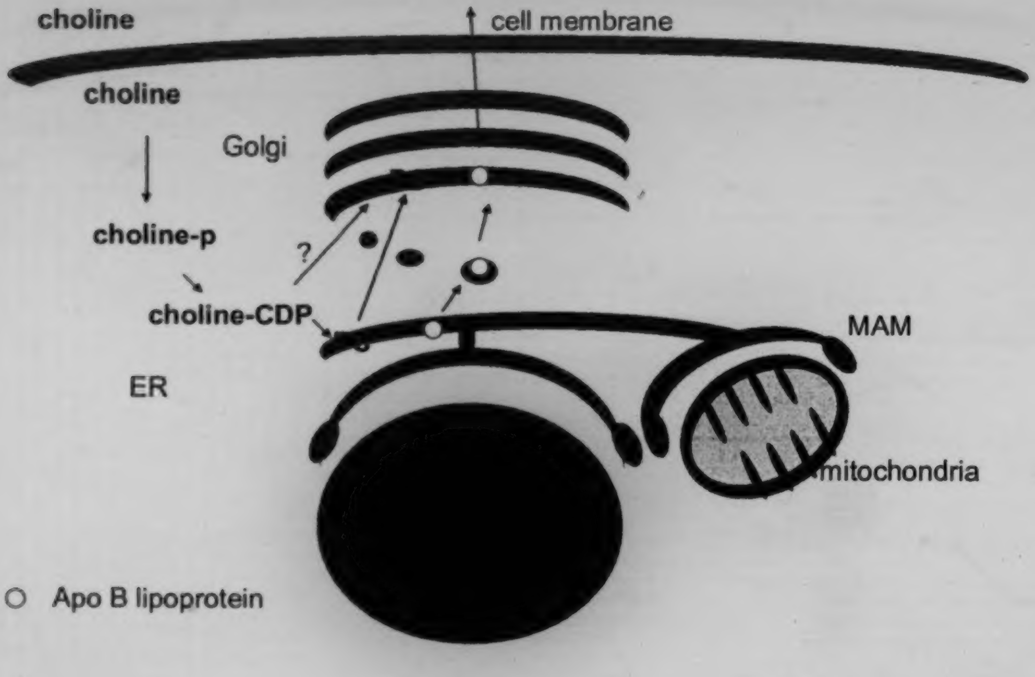
IV.1.3 Hypothesis

The object of this investigation is to determine if there is specific compartmentalization of specific pools of phospholipids to be used in secretion with apo B containing lipoproteins, and to determine the sites within hepatocytes in which PC derived from PS, PE, or via activation of choline (Kennedy pathway) is added to an apo B containing lipoprotein. Based on (Vance and Vance 1986), I hypothesized that PC derived from methylation of PE or via decarboxylation of PS is added early in the secretory process i.e. within the smooth ER or the MAM. PC obtained from the Kennedy pathway may be added during remodeling of the lipoprotein in the Golgi (models of these predictions are shown in Figure IV.1). To begin to test these hypotheses, rats were injected with [^{3}H]choline via the portal vein. The livers were harvested and ER and Golgi isolated. Lumenal apo B was isolated and the lipids associated with apo B were separated by thin layer chromatography (TLC). Radioactivity and PC mass were measured to calculate specific radioactivity and mass of apo B associated PC relative to the protein content of the organelle fraction (dpm or nmol PC/mg organelle protein).

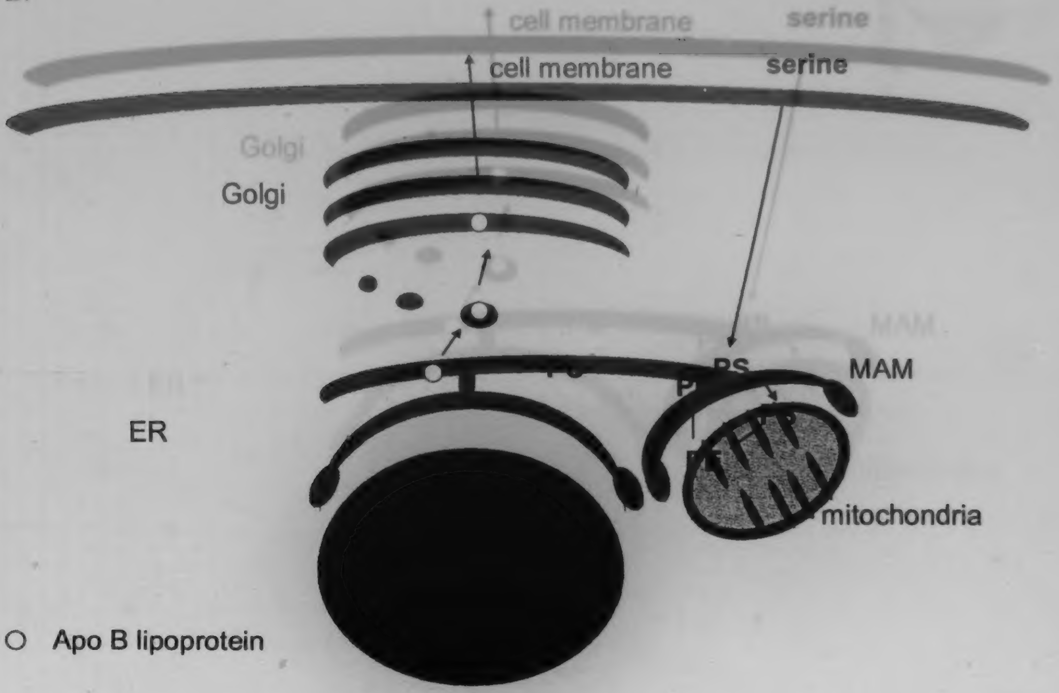
Figure IV.1 Proposed fates of radiolabeled chloline, serine, and ethanolamine in the assembly of an apo B containing lipoprotein

A. choline as precursor, B. serine as precursor and C. ethanolamine as precursor. Question mark in A. denotes possibility of synthesis of PC derived from choline or transport of that PC to the Golgi for incorporation into a lipoprotein. Stricken arrow in C. indicates little of the PE from ethanolamine constitutes the PC in a lipoprotein. White ball, apo B lipoprotein.

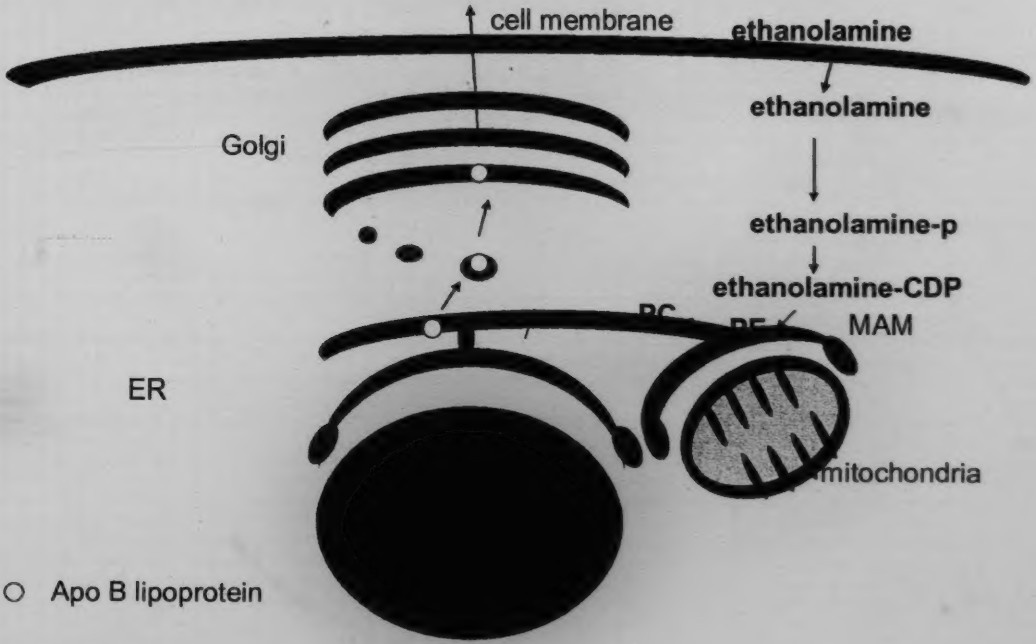
A.



B.


cell membrane
serine
Golgi
PS
MAM
P
ER
mitochondria
○ Apo B lipoprotein

C.


cell membrane
ethanolamine
ethanolamine
ethanolamine-p
ethanolamine-CDP
Golgi
PC
PE
MAM
ER
mitochondria
○ Apo B lipoprotein

As a consequence, portal vein injection of rats with [^{3}H]choline resulted in the isotope being most abundant in PC associated with Golgi lumenal apo B relative to ER lumenal apo B. More PC mass was also recovered in Golgi lumenal apo B PC relative to ER. These results suggest that PC derived from choline might be added to apo B lipoproteins in the Golgi although it was not determined if the recovery of apo B from the ER was complete. Further studies are required to establish the contribution and site of PS derived PC addition to apo B containing lipoproteins in the liver. This study could serve as a foundation to determine if compartmentation of phospholipids plays a specific role in apo B lipoprotein assembly and secretion.

IV.2 Results

IV.2.1 Purity of organelle fractions

IV.2.1.1 Enzyme assays indicate relative purity of ER and Golgi fractions

By modification of the subcellular fractionation procedure of Croze and Morre (Croze and Morre 1984), two fractions of ER - ERI (more dense, enriched in rough ER) and ERII (enriched in less dense, smooth ER) - and Golgi vesicles were isolated. The ERI and ERII fractions were then pooled for further analysis. The purity of the organelle fractions was assessed by measurement of the specific activities of marker enzymes: NADPH:cytochrome c reductase (ER marker) and UDP-galactose:N-acetylglucosamine galactosyltransferase (trans-Golgi marker) (Table IV.1). According to these marker enzymes, contamination of the ER fraction by the Golgi marker was less than 11.6%. Cross-contamination of Golgi membranes by the ER membrane marker enzyme was approximately 21.9%. The enrichment of ER based on comparison of cytochrome C reductase activity in the ER relative to the homogenate, was 8 fold. Enrichment of the Golgi marker enzyme (galactosyltransferase) in the Golgi fraction relative to the homogenate was 25-fold. These results suggest that the Golgi may be more contaminated and less enriched than has been previously reported (Rusinol et al. 1993).

Table IV.1 Organelle Purity based on enzyme marker activity

A rat liver homogenate, ER and Golgi were isolated from a single liver and marker enzyme assays were performed as described in chapter II. Values are averages of three measurements +/-S. D. Enrichment refers to specific activity relative to that of the homogenate. [^{3}H[gal, tritiated galactose.

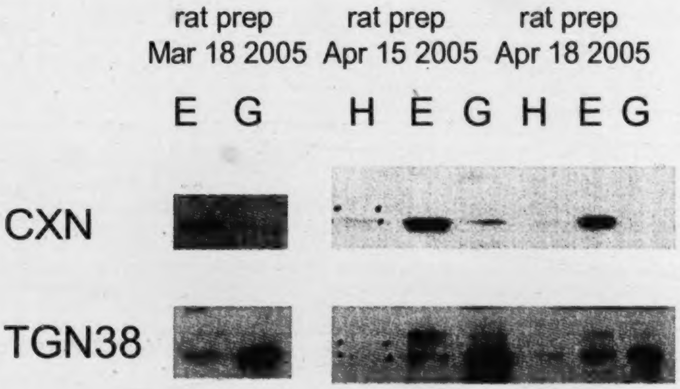
	Cytochrome C reductase activity			Galactosyl transferase activity		
	nmol/min/mg cytochrome C reduced	%ER activity	Enrichment	nmol/h/mg [^{3}H]gal incorporated	%Golgi activity	Enrichment
homogenate	1.33+/-0.33	13.19	-	3.76+/-1.22	3.94	
ER	10.11+/-0.51	-	8 fold	11.04+/-4.32	11.56	3 fold
Golgi	2.24+/-0.38	21.9	1.7 fold	95.41+/-15.870	-	25 fold

IV.2.1.2 Western blot analysis also indicates relative purity of ER and Golgi

Samples of homogenate, ER and Golgi were also assessed by Western blot to determine organelle purity. Antibodies against specific intracellular marker proteins were used to identify the various fractions (Figure IV.2). Fractions obtained appeared relatively pure based on the protein markers CXN and TGN38 which identify ER and Golgi microsomes, respectively.

Figure IV.2 Organelle purity determined by Western blot

ER and Golgi were isolated by sedimentation and density ultracentrifugation. Equal amounts of protein from homogenate (H), ER (E) and Golgi (G) were loaded onto 10% (w/w) SDS-PAGE gels, resolved by electrophoresis and transferred to a PVFD membrane as described in chapter II. The membrane was then probed for the presence of the ER protein calnexin (CXN) and the Golgi marker trans-Golgi network protein 38 (TGN38). Samples from three different liver preparations are shown.



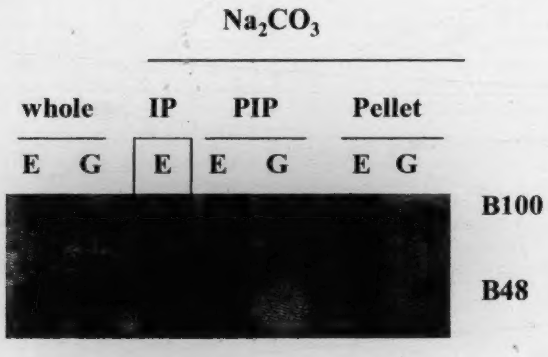
IV.2.2 Apo B can be recovered from ER and Golgi lumen

Initial experiments yielded little PC mass or radioactivity associated with the apo B immunoprecipitation from the lumenal contents after carbonate extraction (Table IV.2; data not shown, see also Figure IV.5, discussed in Section IV.2.5). One explanation for these results might be that the immunoprecipitation was incomplete, or that the carbonate extraction was not complete such that the contents of the ER and Golgi lumena were not released. Alternately, there might have been a problem with the lipid extraction and resolution by TLC. Finally, there might be too little mass or radioactivity associated with apo B to detect using these methods.

To test the first possibility, small scale immunoprecipitation of apo B was performed on ER and Golgi lumenal contents after carbonate treatment. Samples were resolved by SDS-gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. The results are shown in Figure IV.3. The antibody preparation was able to detect apo B in whole ER and Golgi, as well as in the lumenal contents of the ER and membrane fractions of the ER and Golgi after carbonate treatment, the latter of which has been observed by others (Bostrom et al. 1986; Davis et al. 1990; Davis et al. 1990; Wilkinson et al. 1992; Adeli et al. 1997; Hebbachi and Gibbons 2001; Tran et al. 2002; Gusarova et al. 2003) although this result does not preclude the possibility that the carbonate extraction was incomplete (see below). The immunoprecipitation seemed to be complete since there was not any detectable apo B in the lumenal contents after immunoprecipitation and removal of immunocomplexes by Protein A Sepharose. Therefore it appears that incomplete immunoprecipitation is not the reason for the low recovery of PC mass associated with apo B in the ER and Golgi lumen.

Figure IV.3 Apo B can be immunoprecipitated from the lumenal contents of the ER and Golgi. Small scale carbonate extraction was performed on 2 mg of ER or Golgi. Samples were resuspended in buffer then made to 0.1 M sodium carbonate, final concentration. The samples were incubated for 30 min at room temperature. The samples were neutralized to pH 7 and bovine serum albumin added as per (Bostrom et al. 1986). The samples were then incubated with anti-apo B antisera overnight followed by Protein A sepharose to capture the immunopcomplexes. The samples

were treated with sample buffer and loaded onto 5% SDS PAGE along with control lanes of untreated ER and untreated Golgi, as well as concentrated post immunoprecipitate supernatant. The proteins on the gel were then transferred to a PVDF membrane, blocked and probed with anti-apo B. **E**, ER; **G**, Golgi; **whole**, aliquots of organelle fractions before carbonate; **IP**, portion of apo B immunoprecipitated from the lumenal contents of ER after carbonate, highlighted in the boxed region; **PIP**, the lumenal contents after immunoprecipitation with anti-apo B, **pellet**, membrane pellet after carbonate treatment. The results shown are of a single experiment.

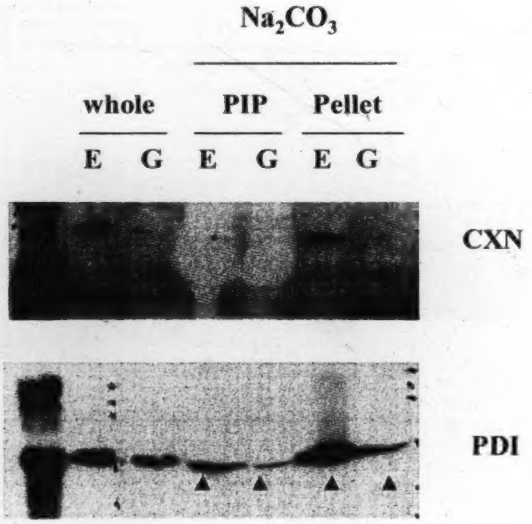


IV.2.3 Efficiency of carbonate treatment optimized

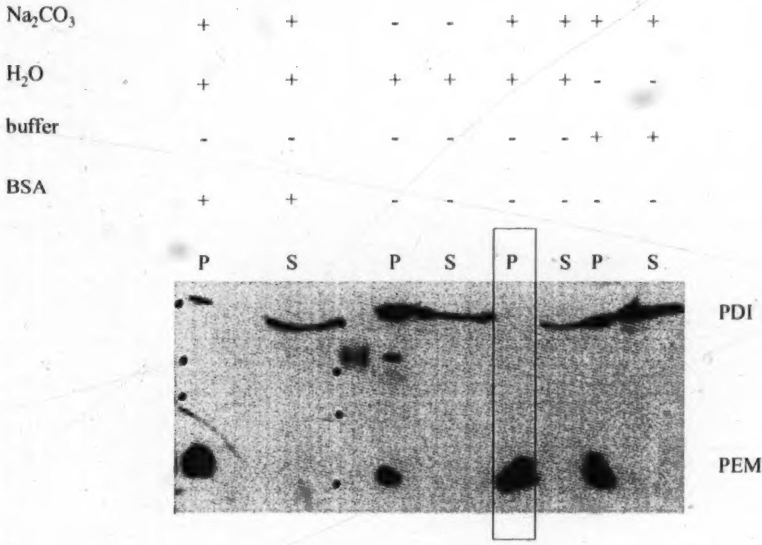
Small scale carbonate extractions of samples of ER were performed to determine the optimum conditions for removal of lumenal protein from membrane-associated proteins. Recovery was assessed by Western blot of markers for ER (Figure IV.4). Initial experiments showed that CXN, a 90 kDa protein component of the ER membrane, remained associated with the membranes after carbonate treatment, as expected. PDI, a 60 kDa ER lumenal protein, however, was also present in the membrane fraction, while very little was recovered in the lumenal fraction, indicating that the carbonate treatment was not sufficient to release the lumenal contents of the ER microsomes (Figure IV.4 A.). Conditions were manipulated to optimize carbonate release by changing the concentration of carbonate, including and excluding albumin (used as a carrier to facilitate protein recovery) or buffer based on (Bostrom et al. 1986) and (Fujiki et al. 1982). It appeared that either 0.1 or 0.2 M carbonate was sufficient to release PDI from the ER lumena while the presence of bovine serum albumin and/or buffer interfered with the release (Figure IV.4 B.). The reason for this result is unknown.

Figure IV.4 Optimization of carbonate extraction of ER and Golgi fractions.
A. Carbonate extraction was incomplete. Conditions were as in Figure IV.3 except that the samples were resolved on 12% SDS-PAGE in order to detect CXN and PDI as markers of membrane and lumenal contents, respectively. Gel legend, as per Figure IV.3. Upward pointing arrows indicate that PDI is largely still membrane associated i.e., not released from the ER or Golgi by carbonate treatment; dark grey arrows, ER; light grey arrows, Golgi. B. Carbonate extraction is optimal when 0.1 M sodium carbonate is incubated on ice and no buffer or albumin is included (designated by boxed in region). The ER was divided into 0.1 mg aliquots ER protein in 400 ul volumes and subjected to conditions specified. The membranes were pelleted and supernatants concentrated by trichloroacetate and deoxycholate. Samples were resolved by SDS-PAGE, transferred to PVDF membrane and probed for PEMT (also an ER membrane protein) and PDI.

A.


Na_2CO_3
whole
PIP
Pellet
E G E G E G
CXN
PDI

B.


Na_2CO_3 + + - - + + + +
H_2O + + + + + + - -
buffer - - - - - - + +
BSA + + - - - - - -
P S P S P S P S
PDI
PEMT

IV.2.4 PC mass associated with ER and Golgi lumenal apo B

In a preliminary experiment, PC mass associated with (i) apo B immunoprecipitated from the ER and Golgi lumen, (ii) the lumenal contents after immunoprecipitation, and (iii) the membrane pellets was determined. More PC was associated with the apo B in the Golgi than in the ER lumen. For both organelle fractions, more PC mass was found in the post-immunoprecipitation lumenal contents while the majority was in the membrane pellets (Table IV.2). While it was expected that the membrane fractions would contain the majority of the PC mass (see Table I.3 in Chapter I), it appeared that there was still some PC in the ER and Golgi cisternae. This result might suggest insufficient antibody was used to immunoprecipitate the apo B or that PC exists in the lumen perhaps as a poorly lipidated particles (Maric et al. 2005) or as a non-apo B associated lipid droplet (Kulinski et al. 2002).

Table IV.2 PC mass associated with ER and Golgi lumenal apo B

The ER and Golgi were isolated from the homogenate of 2 rat livers combined and treated with carbonate to release the lumenal contents. The membranes were pelleted and the supernatant neutralized. Anti-apo B immunoprecipitation was performed as described in Chapter II. Lipids were extracted and resolved by TLC. The PC bands matching the location of a standard PC were scraped, digested with perchlorate and PC mass was determined with Malachite Green phosphorous assay. Apo B immunoprecipitation of the lumenal contents, apo B IP; post-immunoprecipitate lumenal contents, post-IP; and membrane pellets after carbonate extraction, pellet. Lipids extracted from a portion of untreated ER (whole) were also analyzed in conjunction with the carbonate treated samples. Results shown are from a single experiment.

Fraction	sample	Mass of PC per organelle protein (nmol PC/mg protein)
ER	apo B IP	0.4
	post-IP	0.3
	pellet	66.9
	whole	154.2
Golgi	apo B IP	3.2
	post-IP	1.7
	pellet	51.7

IV.2.5 Little [^{3}H]choline is recovered in the lumenal apo B fraction after portal vein injection

A number of experiments was performed in which a rat liver was infused with 400 uCi [^{3}H]choline for varying times. Little of the radiolabel was recovered in the PC associated with the isolated apo B containing lipoproteins overall, but more was found associated with the Golgi apo B than with the ER apo B (for example see Figure IV.5). The radiolabel is primarily recovered in the membrane pellets which might be expected given the mass of PC present in this fraction. However, overall, much of the label was not recovered during isolation of the organelles nor after lipid extraction (Table IV.3; data not shown).

Figure IV.5 Little radioactive PC is associated with ER lumenal apo B, more with the Golgi lumenal apo B.

400 uCi [^{3}H]choline was injected into the portal vein of an anesthetized rat and the liver harvested 30 min later for isolation of ER and Golgi, carbonate extraction, apo B immunoprecipitation and lipid processing as described in Chapter II. Lipids were extracted from membrane, apo B immunoprecipitates, and post-immunoprecipitate lumenal contents, cold phospholipid carrier added, and the phospholipid resolved by TLC. PC bands were scraped into scintillation vials, fluor added, and the

radioactivity associated with the PC measured. Results are from a single experiment but are similar to separate experiments in which [^{3}H]choline was injected for 8 and 15 min (not shown). **pellet**, membrane pellet; **apo B**, radioactivity associated with anti-apo B immunoprecipitation of lumenal contents; **aft IP**, radioactivity associated with lumenal contents after anti-apo B immunoprecipitation

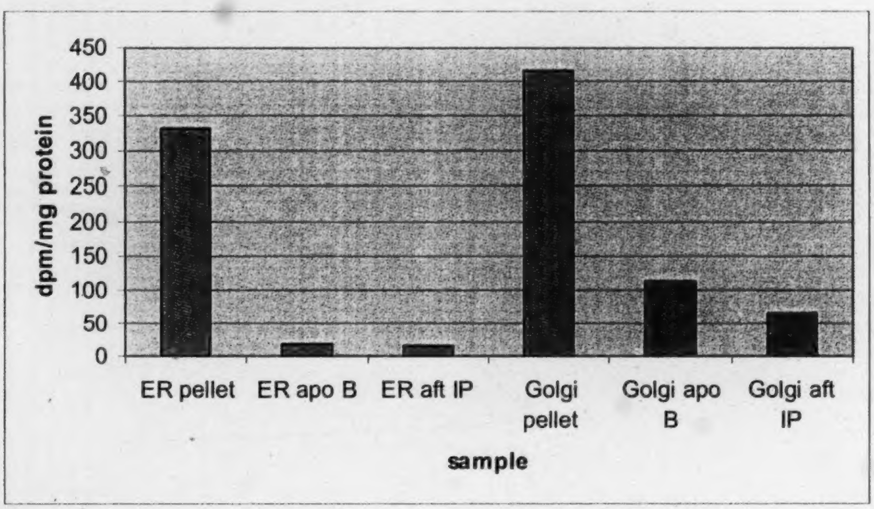


Table IV.3 Total radioactivity recovered per milligram organelle protein prior to carbonate and lipid extraction with or without choline deprivation.

400 uCi [^{3}H]choline was injected into the portal vein of an anesthetized rat, and approximately 30 min later, the animal was sacrificed. The ER and Golgi were isolated and aliquots of purified organelle fractions were taken for quantification of radiolabeling. Results of the choline deprivation are the combination of 2 rats injected with 400 uCi [^{3}H]choline each. (Effects of choline deprivation are discussed in the next Section).

sample	condition	dpm/ mg organelle protein
ER total	Choline sufficient	8123
Golgi total		18752
ER total	Choline deprivation	60402
Golgi total		122803

IV.2.6 Effects of choline deprivation

While the little amount of radiolabel detected in previous experiments might reflect limited recovery of the organelles during isolation, it might also be that the [^{3}H]choline is unable to be incorporated because it represents so little of the choline pool available in the cell for PC synthesis and subsequent incorporation into an apo B containing lipoprotein. To test if this were a possibility, the intracellular pool of choline was depleted by depriving rats of dietary choline for 22 days then the experiments described above were repeated. Aliquots before and after immunoprecipitation of the lumenal contents were taken to determine the radioactivity before and after lipid extraction to determine the efficiency of the immunoprecipitation (Figure IV.6) and the lipid extraction procedure (Table IV.4). The post-carbonate, post-immunoprecipitation ER and Golgi lumenal fractions were also treated with fumed silica (Cabosil) to recover any lumenal PC in the event the immunoprecipitation was incomplete or PC was associated with lipid poor particles or the TG rich lumenal droplet described above. Cabosil has been used to concentrate lipoproteins after density gradient centrifugation for lipid extraction and SDS-PAGE/Western blot in other investigations (Vance et al. 1984; Vance and Vance 1986; McLeod et al. 1994). Prior to carbonate treatment, greater incorporation of the isotopically labeled choline into the ER and Golgi fractions was observed under choline deficient compared to choline sufficient conditions, with more labeling observed in the Golgi than the ER in both instances (Table IV.3). This result indicates that the choline pool was depleted, thus increasing radioactivity of the choline pool and increasing ability of the radiolabel to contribute to phospholipid production. After carbonate treatment and apo B immunoprecipitation, more [^{3}H]choline was incorporated into Golgi than ER membranes and lumenal total phospholipids before and after lipid extraction (Table IV.4), and into Golgi lumenal apo B in particular, similar to results observed above. However, in the carbonate treated ER, the radioactivity associated with the Cabosil after immunoprecipitation exceeded that recovered from the apo B immunoprecipitation. It was not clear until the Cabosil fractions were analyzed by Western blot if this was due to incomplete immunoprecipitation (Figure IV.6). Apo B immunoprecipitation from the ER lumena was therefore incomplete. Thus, much more antibody is required. Other protein components of the ER might also have interfered with the recovery of the apo B containing lipoproteins. Alternately, or in addition, little PC derived from choline is added to an apo B containing lipoprotein in the ER, in congruence with results obtained by other investigators (Howell and Palade 1982; Janero and Lane 1983; Higgins and Hutson 1984; Higgins and Fieldsend 1987). Measurement of PC mass associated with apo B, the post-immunoprecipitation lumenal contents and membrane pellets for ER and Golgi for this experiment was not determined.

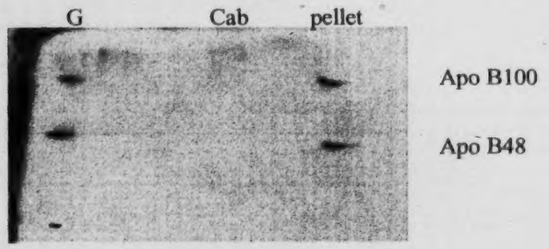
Table IV.4 Effects of choline deprivation

Two rats were deprived of dietary choline for 22 days. Each liver was injected with 400 uCi [^{3}H]choline for 30 min and the livers pooled before harvesting the ER and Golgi. Carbonate treatment was followed by apo B immunoprecipitation of the ER and Golgi lumena. Aliquots of the carbonate fractions were collected in order to measure incorporation of [^{3}H]choline into total phospholipid prior to and after lipid extraction to determine if the low recovery seen in Figure IV.5 and phospholipid mass (Table IV.2) was due to the method of lipid extraction. **bef IP**, radioactivity associated with lumenal contents before immunoprecipitation with anti-apo B polysera; **apo B**, radioactivity associated with anti-apo B immunoprecipitation of lumenal contents; **aft IP**, radioactivity associated with lumenal contents after anti-apo B immunoprecipitation; **Cab**, radioactivity associated with treatment of the lumenal contents with Cabosil after immunoprecipitation; **aft Cab**, radioactivity of lumenal contents remaining after immunoprecipitation and Cabosil treatment; **pellet**, membrane pellet; **whole**, ER or Golgi before carbonate treatment; **N/A**, not available

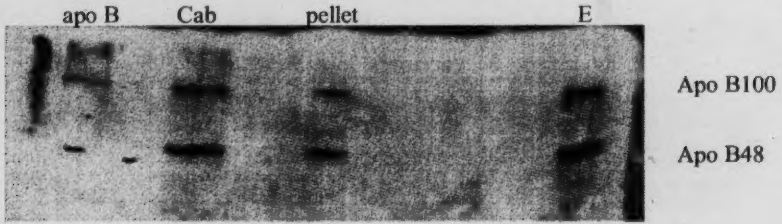
Radioactivity associated with PC (dpm/mg organelle protein)		
sample	before lipid extraction	after lipid extraction
ER bef IP	12635	1526
ER apo B	N/A	40
ER aft IP	10682	1106
ER Cab	N/A	1230
ER aft Cab	11424	753
ER pellet	23421	24698
ER whole	12083	85247
Golgi bef IP	29011	5221
Golgi apo B	N/A	2775
Golgi post IP	20403	2662
Golgi Cab	N/A	409
Golgi aft Cab	18066	757
Golgi pellet	46285	37882
Golgi whole	245606	99762

Figure IV.6Apo B immunoprecipitation from the ER lumen is incomplete

Conditions and sample legend as for Table IV.4 **Top panel**, ER; **Bottom panel**, Golgi Portions of the apo B immunoprecipitation (apo B) and Cabosil treatment (Cab) were not lipid extracted but instead were used to detect apo B by Western blot. **E**, sample of whole ER, not treated with carbonate; **G**, sample of whole Golgi, not treated with carbonate. Results are from a single experiment.



Golgi



ER

IV.3 Discussion

IV.3.1 Summary

Initial studies to determine the site and source of PC in an apo B containing lipoprotein were conducted. ER and Golgi fractions were obtained from rat liver after the rats had been injected via portal vein with [^{3}H]choline. The organelle fractions were extracted with carbonate to release the lumenal contents from the membrane-associated lipids and proteins. Apo B containing lipoproteins were then resolved from the lumenal contents and analyzed for radioactivity associated with the PC. The mass of PC associated with apo B was determined. Results to date suggest that PC associated with apo B derived from choline is added to the lipoprotein in the Golgi but could not preclude that the recovery of apo B from the ER was complete.

IV.3.2 Tracking apo B phospholipidation in the secretory pathway of the hepatocyte

An investigation of the role of phospholipids in lipoprotein assembly and secretion is important since phospholipids, particularly PC, are prominent components of all plasma lipoproteins including atherogenic apo B containing lipoproteins. In the liver, PC is made in liver by the CDP-choline pathway and by the methylation of PE. PE can be synthesized from ethanolamine or from decarboxylation of PS. Most enzymes for phospholipid synthesis have been localized to the ER or MAM but the Golgi membranes also possess some of the enzymes necessary for the synthesis of PC (Higgins and Fieldsend 1987; Vance and Vance 1988). MTP and phospholipid transfer protein, which are capable of transferring phospholipid, have also been detected in the Golgi (Jiang et al. 2001; Levy et al. 2002; Swift et al. 2003). Howell and Palade reported that injection of rat liver with either [^{14}C]choline (to label PC), [^{3}H]myo-inositol (to label PI), or [^{33}P]orthophosphate (to label all phospholipids), resulted in 80% of the label in Golgi membranes while 20% was transferred from the Golgi membrane to the lumenal contents which contained a relatively large amount of secretory lipoproteins (Howell and Palade 1982). The authors proposed that an active exchange process of polar lipids between Golgi membranes and Golgi secretory lipoproteins occurs as an explanation for these findings (Howell and Palade 1982).

Given that some investigators have observed addition of lipid to VLDL in the Golgi (Janero and Lane 1983; Bamberger and Lane 1990; Tran et al. 2002; Valyi-Nagy et al. 2002) it is possible that phospholipid is added in the Golgi as well. Alternately, specific utilization and localization of the different sources of phospholipid might occur. Thus PC derived from choline may be added to a lipoprotein in the Golgi as might be suggested by the work done in this thesis. PC from other sources may be added in the ER.

It is also possible that smooth ER, which is observed to contain VLDL like particles by electron microscopy (Alexander et al. 1976), may be contaminating the Golgi and therefore give a "false positive" result indicating that the bulk of phospholipid addition occurs in this organelle. Rough ER and smooth ER have been isolated from rabbit hepatocytes (Cartwright and Higgins 1995; Cartwright et al. 1997). The results from these studies indicate that very small amounts of neutral lipids and cholesterol co-localize with apo B in the lumen of the rough ER. The bulk of the VLDL lipids were found in the lumen of the smooth ER. However, some newly synthesized TG, phospholipid, cholesterol, and cholesteryl ester were also transferred to the lumen of the rough ER and were "chased" into the smooth ER lumen. The different outcomes observed from the various experiments discussed in the introduction of this chapter might reflect differences in isolation techniques and the source of material (rat liver, rat hepatocytes, estrogen treated chicken hepatocytes, rabbit hepatocytes).

IV.3.3 Choline-derived PC may be added to the apo B lipoprotein in the Golgi

Results suggest the addition of choline-derived PC to apo B lipoprotein (depicted in Figure IV.1 A.), i.e., within the Golgi. However, so little radioactivity and mass were obtained - even after choline deprivation – that the experiments need to be repeated. Choline uptake and distribution throughout the liver from the portal vein is rapid, occurring within 2 minutes (Y. Z. Li, personal communication), therefore the results observed may not be due to impaired uptake of choline by the liver.

Another approach to determine the source of apo B phospholipid and sites of addition may be warranted. For example, pharmokinetic manipulation of primary hepatocytes with reagents such as with brefeldin A and or nocadazole to block the secretory pathway followed by isolation of apo B is a possible approach to determine what and when phospholipids are added to apo B. A caveat to this approach, however, is that these compounds have pleiotropic effects which might complicate interpretation of the data.

Alternately, pulse chase studies with primary hepatocytes and dual labeling of protein and lipid could be used. Liver specific knock out of murine CCTα resulted in reduced secretion of apo B and PC (Jacobs et al. 2004). The mechanism underlying this phenomenon is currently under investigation (R. Jacobs, personal communication). It might be of interest to extract the organelle fractions of the knock out liver or from primary hepatocytes to determine the quantity/composition of these lipoproteins. It is possible that the effect is indirect and that apo B is degraded either in the ER compartment by the proteasome, or in a post-ER compartment in order to maintain cellular PC levels in the absence of CCTα. Choline deprivation in mouse did not reduce apo B secretion (Kulinski et al. 2004). Knockout of murine PEMT also reduces apo B secretion when mice are challenged with high fat high cholesterol diet (Noga et al. 2002; Noga and Vance 2003). The mechanism for this effect is also currently under investigation (Y. Zhao, personal communication). The availability of knock out mice, or the ability to knock down enzymes involved in phospholipid synthesis may be a lucrative avenue of investigation with respect to apo B secretion by the liver.

IV.3.4 Projected outcomes with ethanolamine or serine precursors

The other pathways for the synthesis of PC were not investigated in this thesis. It might prove fruitful to investigate the contribution of ethanolamine via the CDP-ethanolamine pathway and PS decarboxylation to PE that is methylated to form PC incorporated into apo B containing lipoproteins along the secretory pathway using whole liver and portal vein injection/time course as initially planned. The results expected, if compartmentation and preferential utilization of serine for PC synthesis occur, are shown in Figure IV.1 B. and C. Thus, little radioactivity from [^{3}H]ethanolamine would be expected to be recovered in the PC fraction of apo B containing lipoproteins found in either the ER or the Golgi (Figure IV.1 B.). Instead, serine would be exchanged for PC or PE in the ER or MAM, the lipid transported to the mitochondria where it would be decarboxylated to form PE, returned to the ER for methylation and subsequently incorporated into an apo B containing lipoprotein. This event would be expected to occur early in the secretory pathway. Evaluation of the contribution of serine is further complicated because, in addition to serine being a precursor of the head-group of PS, PE, and also of PC (via methylation of PS-derived PE), radiolabel is likely to be incorporated into the acyl chains and the glycerol backbone of these phospholipids (Yeung and Kuksis 1976; Vance and Vance 1986). Therefore, PS, PE and PC will have to be further digested with phospholipase C to determine the distribution of radiolabel between the head-group and diacylglycerol moieties of these phospholipids. Recently, PSS1 and PSS2 knock out mice have been generated and are currently under active investigation in this laboratory. There do not appear to be overt phenotypes in these mice. The plasma lipoprotein profile under chow fed, fasting, or fat feeding has yet to be investigated. If PS is the source of PE for PC incorporation into an apo B containing lipoprotein, it might be expected that absence of PSS enzymes would reduce the amount of lipoprotein secreted. This phenotype may be demonstrable only under fat feeding, similar to PEMT knock out mice.

Conclusion

From the clinical viewpoint, there is much interest in the study of the production of apo B containing lipoproteins such as VLDL and LDL since elevated levels of plasma apo B are strongly correlated to the development of coronary heart disease and observed in the metabolic syndrome and complications associated with diabetes. The regulation and mechanism of apo B containing lipoprotein biogenesis is complex and not entirely understood. In this thesis, the early stages of apo B peptide synthesis were examined, in particular, how the apo B peptide enters or translocates into the lumen of the ER. Second, the initial experiments were conducted to determine the intracellular sites and sources of phospholipid added to the lipoprotein as it is assembled.

Apo B has been found to demonstrate transient transmembrane-like properties, and while the protein possesses many hydrophobic regions, it lacks the canonical 20 amino acid membrane spanning regions present in typical integral membrane proteins. This transmembrane-like property may be due to the presence of pause transfer sequences, PTS, which may confer a pause or delay in the translocation of apo B, although PTS have been observed only *in vitro*. It is unknown if PTS affect the secretion of an apo B containing lipoprotein *in vivo*. In Chapter III of this thesis, a PTS was appended to a typical secretory protein, prolactin (Prl), referred to as Prl-pause, and stably expressed in rat hepatoma cells to determine if the presence of the PTS would result in a distinguishable phenotype such as impaired secretion relative to cells expressing the native protein or a fusion protein in which a "nonsense" sequence was attached (Prl-stuffer). This PTS containing fusion protein demonstrated transmembrane like activity *in vitro*. However, although Prl-pause could be detected in cellular lysates from clones expressing this protein, it could not be detected in the media. Subsequent analyses indicated that the protein forms aggregates in the ER, suggesting that the protein is malformed, although further work is required to verify this result. Therefore it was concluded that this method of approach may not be suitable for determining the role in PTS *in vivo*. Alternately, since limited secretion of PTS-chimeras has also been observed by others, PTS might not play arole in apo B secretion. Recent work in another laboratory established that β sheet regions of apo B, not PTS, determine translocation efficiency of apo B (Yamaguchi et al. 2003; Yamaguchi et al. 2006).

The 3D structure of apo B has been predicted (Richardson et al. 2005; Johs et al. 2006; Krisko and Etchebest 2007). However, the function of specific amino acid sequences within this large, hydrophobic protein relative to lipoprotein assembly and secretion is only beginning to be elucidated. For example, the N terminal 900-1000 amino acids of apo B has been found to be responsible for the initiation of lipoprotein lipidation (Gretch et al. 1996; Shelness et al. 2003; Manchekar et al. 2004; Richardson et al. 2005). Mutagenesis studies have established that N terminal cysteine residues (for disulfide bridge formation and palmitoylation) and glycosylation sites within apo B play roles in lipoprotein secretion (Tran et al. 1998; Zhao et al. 2000; Vukmirica et al. 2002; Vukmirica et al. 2003). Finally, as studied in this thesis and elsewhere, specific sequences affecting translocation into the ER lumen during apo B containing lipoprotein assembly have been investigated (Chuck et al. 1990; Chuck and Lingappa 1993; Rusinol et al. 1998; Yamaguchi et al. 2003;

Yamaguchi et al. 2006). Clearly further studies are required to determine the amino acid sequences of apo B essential for lipoprotein secretion.

In Chapter IV of this thesis, the foundation for investigating the intracellular sites in which phosphatidylcholine (PC) is added to apo B in the liver was initiated. PC is the major phospholipid in lipoproteins secreted by the liver although the mechanism by which the lipid is added to apo B has not been clearly established. One way to begin to delineate this mechanism is to determine the source and sites whereby the PC is added. PC can be synthesized from choline or from methylation of phosphatidylethanolamine (PE). PE itself is synthesized from either ethanolamine or decarboxylation of phosphatidylserine (PS) in the mitochondria. PS is synthesized in the ER or the mitochondria associated membrane (MAM) by serine exchange with PC or PE. Previous results suggested a preferential utilization of PC obtained from choline and methylation of PE derived from decarboxylation of PS for VLDL assembly (Vance and Vance 1986) although the intracellular sites in which the phospholipids are added from the various sources has not been established. The contribution and location of the PC derived from choline to an apo B containing lipoprotein was investigated in this thesis. Portal vein injection of [^{3}H]choline into rat liver was followed by isolation of ER and Golgi after various time points. The lumenal contents of these organelles were recovered and and apo B containing lipoproteins isolated to measure the mass and radioactivity associated with the apo B PC. Results suggested that choline-derived PC is added to an apo B lipoprotein within the Golgi (see Figure IV.1 A.), suggestive of exchange or remodeling of the lipoprotein in this organelle. However, so little radioactivity and mass were obtained that the experiments need to be repeated. Further studies are required to determine the contribution and intracellular localization of the addition of PC derived from methylated PE that has been synthesized from either ethanolamine or from decarboxylation of serine.

The interplay between apo B, lipids and other factors during the lipoprotein assembly process in the liver is only beginning to be discerned. It is hoped that these studies will serve as a foundation for better understanding this process and for the development of therapy to combat disease.

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